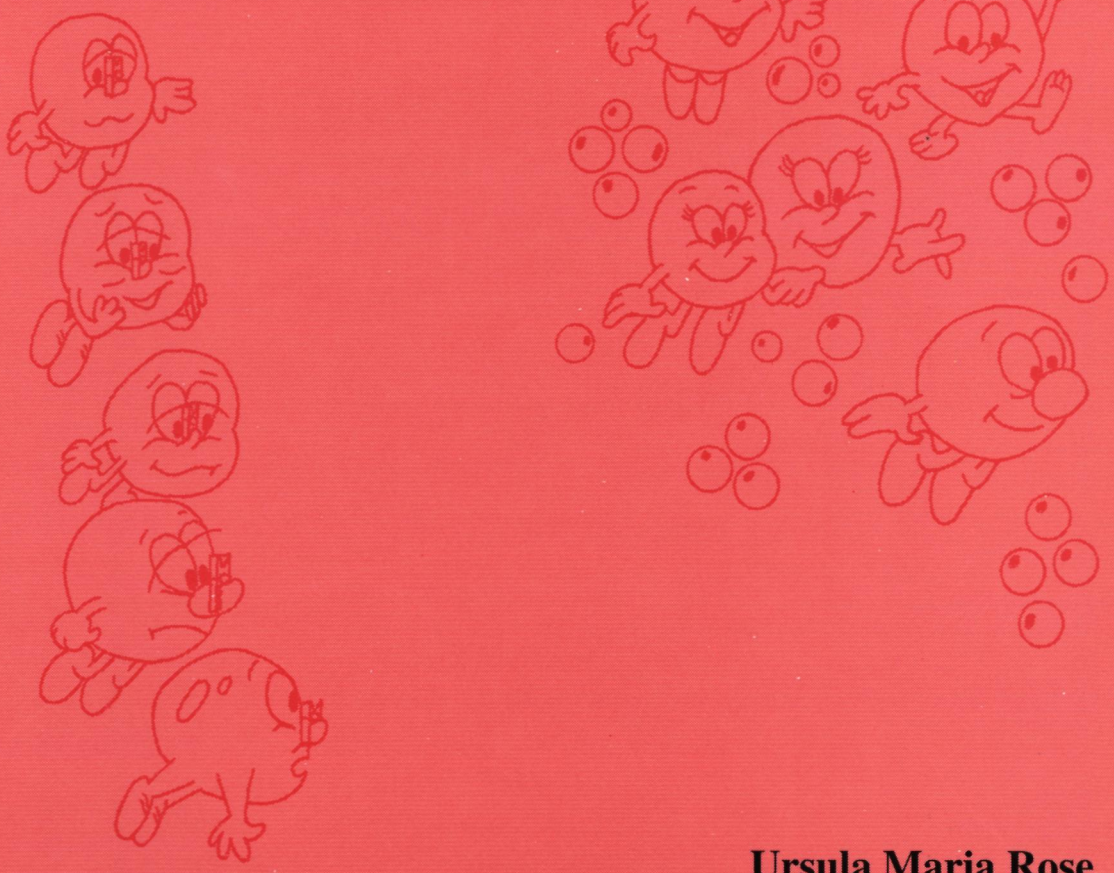
THE ROLE OF INTRACELLULAR CALCIUM IN ANOXIA-INDUCED CELL INJURY IN RENAL EPITHELIAL CELLS



Ursula Maria Rose

THE ROLE OF INTRACELLULAR CALCIUM IN ANOXIA-INDUCED CELL INJURY IN RENAL EPITHELIAL CELLS

THE ROLE OF INTRACELLULAR CALCIUM IN ANOXIA-INDUCED CELL INJURY IN RENAL EPITHELIAL CELLS

een wetenschappelijke proeve op
het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholicke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op woensdag
22 juni 1994, des namiddags te 1.30 uur precies

door

URSULA MARIA ROSE

geboren op 13 januari 1966 te Deurne

Promotor:	prof. dr. C.H. van Os
Co-promotores:	dr. R.J.M. Bindels
	dr. J.W.C.M. Jansen (Solvay Duphar BV, Weesp)

The studies presented in this thesis were performed at the Department of Cell Physiology, University of Nijmegen, The Netherlands.
This thesis was financed by Solvay Duphar BV, Weesp, The Netherlands.

Voor mijn ouders

drukkerij: OMI Offset, Utrecht

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Rose, Ursula Maria

The role of intracellular calcium in anoxia-induced cell injury in renal epithelial cells / Ursula Maria Rose. - [S.l. : s.n.]. - Ill.
Thesis Nijmegen. - With ref. - With summary in Dutch.
ISBN 90-9007078-8
Subject headings: ischaemia / kidneys / anoxia.

ABBREVIATIONS

ATP	adenosine 5'-triphosphate
AVP	vasopressin
BCECF-AM	2',7'-bis-(-2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester
BSA	bovine serum albumine
bPTH, 1-34	bovine parathyroid hormone
Ca^{2+}	calcium
Ca^{2+}_o	extracellular calcium
$[Ca^{2+}]_o$	extracellular calcium concentration
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	cyclic adenosine monophosphate
CCCP	carbonyl cyanide-m-chlorophenylhydrazone
CCD	charge coupled device
CT	calcitonin
cTAL	cortical thick ascending limb of the loop of Henle
D600	meyhoxyverapamil
EGTA	ethylene glycolbis(ß-aminoethyl ether)-N.N.N'.N'-tetraacetic acid
FCS	foetal calf serum
Fura-2 AM	fura-2 acetoxymethyl ester
IBMX	3-isobutyl-1-methylxanthine
HEPES	4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid
KHB	Krebs-Henseleit buffer
LDH	lactate dehydrogenase
mTAL	medullary thich ascending lib of the loop of Henle
NADH	nicotinamide adenine dinucleotide
NPS	Newcastle Photonic System
pH_i	intracellular pH
pH_o	extracellular pH
PGE_2	prostaglandin E_2
PT	proximal tubule
Tris	tris(hydroxy)methylaminomethane

CONTENTS

CHAPTER 1

General Introduction

Renal ischemia involves cessation of perfusion of the kidney leading to oxygen and substrate deprivation (Weinberg, 1991). Depending on the severity of renal ischemia, hypoperfusion is associated with a graded vascular and tubular injury ranging from none to substantial cellular necrosis. As a result, kidney function is suppressed which will eventually result in renal failure (Brezis et al., 1991). In figure 1, the effects of vascular and tubular injury are summarized. Since ischemic renal failure is a substantial clinical problem, it has been a research subject for many years. However, in spite of the many attempts that have been made to understand hypoxic injury, the basic mechanisms responsible for organ failure have remained elusive. Especially the heterogeneous responses of the various cell types within the kidney raised many difficulties, which have not been sorted out at the moment.

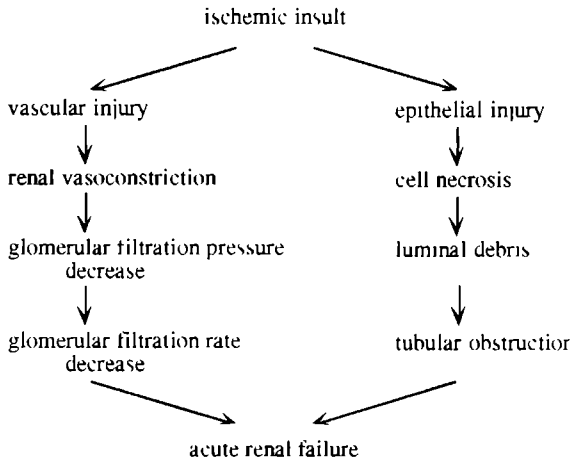


Figure 1
The effects of ischemia on vascular and tubular kidney tissue which eventually leads to acute renal failure (adapted from Schrier et al., 1987).

Kidney structure

The mammalian kidney is characterized by an intra-organ heterogeneity in structure, metabolism and function. Under normal physiologic conditions this complexity is maintained by the functional integrity of the microvasculature of the organ, which depends on the balance of vasoconstrictive and vasodilatory factors (Bonventre, 1993). However, whenever this balance is disturbed, an inadequate or absent blood flow can lead to ischemia and ischemia-induced injury (Weinberg, 1991; Bonventre, 1993). Because of the heterogeneous blood

flow in the kidney, hypoperfusion causes intrarenal gradients of ischemia, which leads to heterogeneity in injury along the nephron (Humes, 1986). In addition, the response of the kidney to ischemia is heterogeneous due to the specific biochemical properties of the various tubular segments along the nephron. In this respect, the nephron, which is the functional unit of the kidney, can be divided into four main tubular segments: the proximal tubule, the loop of Henle, the distal and the collecting system (Fig. 2). Each of these segments plays a defined role in the formation of urine. The initial stage of urine formation is the filtration of plasma and the accumulation of the ultrafiltrate in the lumen of Bowman's capsule. Filtration in the glomerulus is so extensive that 20-30% of the water and solutes are removed from the plasma that flows through it. Next, the glomerular filtrate is passed through the renal tubule. The proximal tubule initiates the process of reducing the volume of the glomerular filtrate by absorbing a major fraction of the filtered water and solutes isosmotically. About 70% of the filtrate is reabsorbed before the filtrate reaches the loop of Henle. In the thick ascending limb of the loop of Henle, 20% of the filtered NaCl is reabsorbed, which results in dilution of the tubular fluid and generation of a hypertonic interstitial fluid. Finally, in the distal convolution and collecting duct system the final and accurate adjustment of electrolyte and acid-base excretion is realized, which results in the production of urine (Beck et al., 1992).

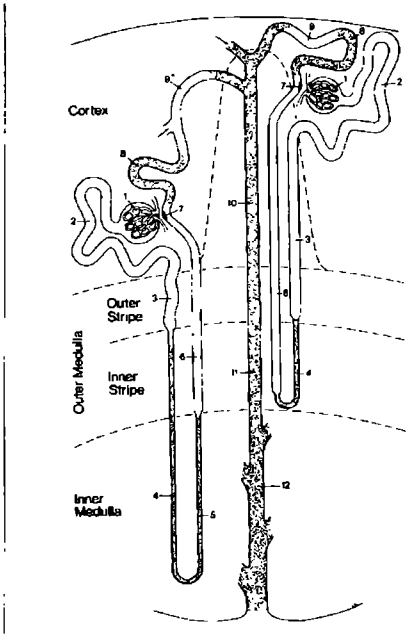


Figure 2
Tubular segments of the mammalian kidney nephron in the cortical and medullary region.
1. glomerulus
2. proximal convoluted tubule
3. proximal straight tubule
4. descending thin limb
5. ascending thin limb
6. thick ascending limb
7. macula densa
8. distal convoluted tubule
9. connecting tubule
9*. connecting tubule of the juxtamedullary nephron
10. cortical collecting duct
11. outer medullary collecting duct
12. inner medullary collecting duct

(adapted from Kriz et al., 1988)

Ischemic cell injury has been shown to be most pronounced along the proximal tubule, less severe in the thick ascending limb and minimal or absent in the distal segments. As a result, proximal reabsorption is reduced which leads to a decrease in fluid reabsorption. In addition, the concentrating capacity of the loop of Henle is reduced, and electrolyte transport along the distal system is affected (Beck et al., 1992).

Several mechanisms of ischemia-induced tubular injury have been recognized, and have been proposed. The following biochemical events at the cellular level may generate the onset of injury:

Alterations of purine nucleotide metabolism

All tissues have a controlled balance between energy production and utilization. During ischemia however, when this balance is disrupted due to oxygen deprivation, cellular ATP levels fall rapidly (Kehrer et al., 1990). Cells and tissues attempt to compensate for the ATP loss by increasing the glycolytic ATP production (Kehrer et al., 1990; Weinberg, 1991; Bonventre, 1993). Medullary cells in the kidney, for example, are adapted to a relatively anoxic environment even under normal physiologic conditions, and can therefore increase their glycolytic rate whenever necessary (Bagnasco et al., 1985; Uchida et al., 1988). Other kidney regions such as the proximal tubule on the other hand, are not very glycolytic and are not capable to produce sufficient ATP via glycolysis (Bagnasco et al., 1985). As a result of different energy supply capacities under ischemic conditions, different grades of susceptibility to injury exist in the kidney (Donohoe et al., 1978; Brezis et al., 1985; Shanley et al., 1986a, 1986b). Since cellular processes, which include protein synthesis, lipogenesis and membrane transport, critically depend on hydrolysis of ATP, ATP depletion leads to cellular dysfunctioning. As a result, cell injury develops. In addition, reperfusion after an ischemic insult leads to a delay in ATP production since the cellular pool of adenine nucleotides and nucleosides, both needed for the formation of ATP, is reduced due to washout (Osswald et al., 1977). Moreover, return of the ATP levels to normal may also be prevented by high concentrations of Ca^{2+} in the mitochondria, which leads to uncoupling of the oxidative phosphorylation (Rossi et al., 1964). In conclusion, better maintenance of cellular nucleotides during ischemia and recovery of ATP during reperfusion is needed in order to survive an ischemic insult.

Calcium

The disturbance of cell Ca^{2+} homeostasis is another factor involved in ischemia-induced pathophysiological processes. Under normal physiological conditions, $[Ca^{2+}]_i$ is maintained between 50 to 200 nM by various mechanisms (Fig. 3). Since $[Ca^{2+}]_i$ is about 4 orders of magnitude lower than in the extracellular medium (~1 to 2 mM) (Schrier, 1987; Nicotera et al., 1990), a large electrochemical gradient exists across the plasma membrane, which constitutes the driving force for Ca^{2+} influx through voltage-sensitive or receptor-operated Ca^{2+} channels (Siesjö, 1989). Voltage-dependent Ca^{2+} channels have been described in detail for excitable tissues such as the cardiac muscle and skeletal muscle. In non-excitable tissues such as the renal epithelium, however, its presence and properties are largely unknown (Nicotera et al., 1990).

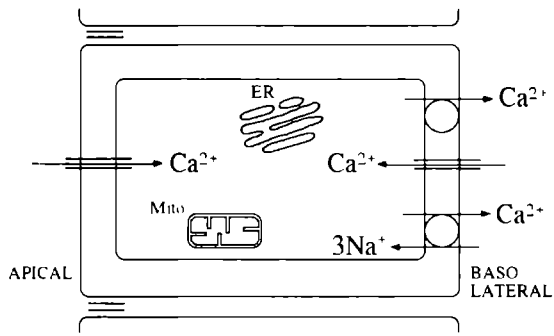


Figure 3

Mechanisms of cellular calcium regulation in renal epithelial cells: Ca^{2+} influx via Ca^{2+} channels in the basolateral and apical membrane, Ca^{2+} efflux via Ca^{2+}-ATPases and the Na^+-Ca^{2+} exchanger in the basolateral membrane, and Ca^{2+} sequestering in the endoplasmic reticulum (ER) and mitochondria (Mito).

Despite the Ca^{2+} entry pathways, a steep Ca^{2+} gradient exists under normal physiological conditions. Several processes are involved in maintaining this gradient and cell Ca^{2+} homeostasis.

First, the low permeability for Ca^{2+} of the plasma membrane is essential in maintaining cellular Ca^{2+} homeostasis (Siesjö, 1989; Bonventre, 1993).

In addition, the continuous inflow of Ca^{2+} through the plasma membrane is balanced by active Ca^{2+} extrusion from the cytosol through plasma membrane Ca^{2+}-ATPases (Humes, 1986; Carafoli, 1987). Next to these Ca^{2+}-ATPases, Na^+-Ca^{2+} exchangers may be involved in regulating Ca^{2+} homeostasis (Carafoli, 1987) by Ca^{2+} extrusion against cytosolic Na^+. The Na^+-Ca^{2+} exchanger is

indirectly ATP-dependent since the Na^+ gradient used for Ca^{2+} extrusion, is created by Na^+/K^+-ATPase activity (Siesjö, 1989).

Another process involved in Ca^{2+} homeostasis, is Ca^{2+} sequestering into mitochondrial and non-mitochondrial storage sites (Cheung et al., 1986; Humes, 1986; Schrier, 1987; Nicotera et al., 1990). The storage of Ca^{2+} in the endoplasmic reticulum is of importance for Ca^{2+} cell homeostasis (Humes, 1986; Carafoli, 1987). Ca^{2+} uptake takes place through Ca^{2+}-ATPases which exhibit a high Ca^{2+} affinity (Schrier, 1987; Nicotera et al., 1990). In contrast, Ca^{2+} uniporter carriers in mitochondria have a low affinity for Ca^{2+}, which implies that under resting conditions, mitochondria play a minor role in buffering cytosolic Ca^{2+} (Carafoli, 1987; Schrier, 1987; Nicotera et al., 1990).

At physiological conditions, Ca^{2+} fulfils important roles. For example, extracellular Ca^{2+} stabilizes the plasma membrane structure (Nicotera et al., 1990), and intracellularly it modulates enzymes in metabolic pathways (Siesjö, 1989), and it serves stimulus-contraction and stimulus-secretion coupling (Siesjö, 1989). All these functions require that Ca^{2+} is released from intracellular stores, or that Ca^{2+} influx is started by the opening of membrane channels in response to cell activation by transmitters or hormones (Siesjö, 1989). However, during energy-deprived states such as ischemia, Ca^{2+} homeostasis fails leading to Ca^{2+} overload which causes disturbance of normal cell functioning and threatens cell viability.

Ca^{2+} overload is a characteristic phenomenon in necrotic cells (Humes, 1986; Weinberg, 1991; Bonventre, 1993). Cellular Ca^{2+} overload can be the result from either enhanced influx of extracellular Ca^{2+} or an impairment of Ca^{2+} extrusion from the cells (Nicotera et al., 1990). Moreover, loss of plasma membrane barrier to Ca^{2+}, allows unrestricted uptake of Ca^{2+} (Weinberg, 1991). If large amounts of Ca^{2+} enter a cell, mitochondria, which exhibit a high Ca^{2+} uptake capacity, become important storage sites (Cheung et al., 1986). Ca^{2+} is taken up by an uniporter driven by an electrical driving force that is generated by the active extrusion of protons along the electron transport system (Humes, 1986; Schrier, 1987; Nicotera et al., 1990), or maintained by ATP hydrolysis (Schrier, 1987) Since the same electro-motive force across the inner mitochondrial membrane is also critical in the initiation of oxidative phosphorylation or ATP synthesis (Humes, 1986; Schrier, 1987; Nicotera et al., 1990; Bonventre, 1993), there is a competition between both processes.

Mitochondria have been shown to selectively transport Ca^{2+} at the expense of ATP production (Humes, 1986; Schrier, 1987; Nicotera et al., 1990). During ischemia, when Ca^{2+} extrusion and sequestering is inhibited due to low ATP availability, $[Ca^{2+}]_i$ increases and mitochondria take up Ca^{2+}. As a result, Ca^{2+} overload develops, which causes uncoupling of the oxidative phosphorylation (Rossi et al., 1964) leading to further ATP depletion. Moreover, increased activity of Ca^{2+}-ATPases that try to reduce the Ca^{2+} overload, increases energy consumption and contributes to additional ATP depletion (Bonventre, 1993).

Mitochondrial and cytosolic Ca^{2+} overload can be detrimental to cells in various ways. High $[Ca^{2+}]_i$, for example, induces disaggregation of microtubuli leading to disruption of the cytoskeleton (Nicotera et al., 1990; Yin et al., 1979). Moreover, Ca^{2+} stimulates degradative enzymes such as phospholipases, catalyzing membrane phospholipid hydrolysis, and proteinases causing proteolysis of, for example, membrane integral proteins and cytoskeletal elements (Farber, 1990; Nicotera et al., 1990; Yin et al., 1979).

During ischemia, cellular Ca^{2+} homeostasis is challenged due to ATP depletion. As a result Ca^{2+} accumulation and cell injury develop. A relevant question is, however, whether Ca^{2+} overload is the primary factor in causing ischemic cell injury, or whether it is the consequence of cell injury. If Ca^{2+} is the primary mediator of ischemia-induced damage, then prevention of Ca^{2+} accumulation is expected to prevent cell injury. In this respect, Ca^{2+} channel blockers have shown to be protective against *in vivo* ischemia-induced renal injury (Burke et al., 1984; Schrier, 1987). Nevertheless, it remains somewhat controversial whether these protective effects are excerted primarily on vascular or on renal tubular cells. Since Ca^{2+} channel blockers had no protective effect in situations of constant renal blood flow, the protection is most likely on vascular instead of tubular cells (Malis et al., 1983). Until now, the presence of voltage-dependent Ca^{2+} channels has not been demonstrated unequivocally in renal epithelial cells.

Cytoskeleton

In addition to the disturbance of the energy balance and Ca^{2+} homeostasis, major structural rearrangements involving the cytoskeleton occur during ischemia (Molitoris et al., 1988, 1989). One phenomenon described in detail is blebbing (Donohoe et al., 1978; Venkatachalam et al., 1978; Lemasters et al., 1987;

Phelps et al., 1989), which is caused by disturbance of the cytoskeleton and cytoskeleton-plasma membrane interaction (Sugrue et al., 1981; Nicotera et al., 1990). The finding that bleb formation in hepatocytes could be mimicked by a Ca^{2+} ionophore and could be prevented by omission of $Ca^{2+}{}_o$, indicated that Ca^{2+} is involved in the formation of surface blebs (Nicotera et al., 1990). In the kidney, blebbing is also closely related to elevated Ca^{2+} (Phelps et al., 1989). Since microfilamental and microtubular structures, both components of the cytoskeleton, are controlled by Ca^{2+}, increases in $[Ca^{2+}]_i$ to the micromolar range lead to disruption of these structures. Such increases in $[Ca^{2+}]_i$ and thus disruption of the cytoskeleton, can occur during ischemia (Yin et al., 1979; Nicotera et al., 1990). Another factor involved in cytoskeletal alterations is ischemia-induced ATP-depletion because ATP is required for polymerization and depolymerization of actine, tubulin and myosin (Bershadasky et al., 1980; 1981; 1983).

Next to structural changes, rearrangement of the cytoskeleton can also lead to functional disorders. Ischemic kidney epithelium for example, looses functional polarity leading to rearrangement of membrane markers. Na/K-ATPase, normally a marker for the basolateral membrane, can also be detected in the apical membrane as a result of ischemia (Molitoris et al., 1988; 1989; 1991; Canfield et al., 1991). In addition, the permeability of tight junctions is increased (Molitoris et al., 1989). These changes indicate a major loss of integrity of the epithelial junctional complex.

With more advanced ischemic injury, vacuoles form within the cell and mitochondria swell. As a result, cells detach from the basement membrane and the cellular debris obstructs kidney tubules (Bonventre, 1993). Disruption of the cytoskeleton as a consequence of ischemia also contributes to cell swelling leading to tubular and venous obstruction (Mason et al., 1989). Prevention of cell swelling can therefore partially protect the kidney against severe injury (Bonventre, 1992; Mason et al., 1989). Since rearrangements of the cytoskeleton during ischemia cause tremendous structural and functional disorders, they still lead to problems during reperfusion: cell debris and cell swelling obstruct the tubular lumen and the blood flow, which leads to a further ischemic period (Donohoe et al., 1978; Mason et al., 1989).

Acidosis

Whole organ ischemia is characterized by decreases in pH_o due to the release of protons during ATP hydrolysis (Hochachka et al., 1983) and enhanced glycolysis (Chan et al., 1982). As a result, enzymes of the glycolytic pathway, such as phosphofructokinase, are inhibited (Bonventre, 1993), leading to further inhibition of ATP production, additional to the abolishment of ATP production via oxidative phosphorylation due to oxygen deprivation. In addition, at very low pH lysosomal protease activity is favoured because of the acidotic pH optima of those enzymes (Barrett, 1980). These processes could lead to cell damage during ischemia.

Low pH, however, can also enhance resistance to the damaging effects of ischemia in whole organs as the kidney (Pentilla et al., 1974; Bonventre, 1984; 1985; Weinberg, 1985; Burnier et al., 1988; Shanley et al., 1988; Zager et al., 1993), or to the effect of metabolic inhibitors in hepatocytes (Kehrer et al., 1990) and kidney (Rodeheaver et al., 1990; Weinberg et al., 1990). The mechanisms behind protection by reduced pH are unknown. Although cellular integrity during ischemia at low pH is improved, ATP levels are still low which argues against a necessary role for ATP preservation in protection (Shanley et al., 1989). One of the possible mechanisms by which acidosis is protective could be prevention of Ca^{2+} overload (Bonventre et al., 1985; Schrier, 1987) by decreasing transmembrane Ca^{2+} fluxes (Altschuld et al., 1981). In addition, phospholipase activity is reduced directly (Schwerz et al., 1983) since the pH optimum is above pH 7.0, and indirectly via reduction of Ca^{2+} availability and Ca^{2+}-calmodulin binding (Busa et al., 1984). Moreover, acidosis stabilizes cell membranes (Bell et al., 1971), leading to improved cell integrity.

Despite all the biochemical events described above, many processes involved in the induction of ischemic injury still remaine elusive. Until now, several models have been used to study mechanisms of cell injury, including chemical anoxia, hypoxia and anoxia.

In chemical anoxia metabolic inhibitors are used to induce ATP depletion. Since in this model absence of oxygen is not a prerequisite, it is more practical than hypoxic or anoxic studies. Another advantage of chemical anoxia is that this model provides complete control over the timing of ATP depletion. Nevertheless, results obtained with chemical anoxia have to be extrapolated with care to the anoxic or hypoxic *in vivo* situation. Although chemical inhibitors

mimick the ATP depletion produced by ischemia, they also posses the potential for additional actions besides ATP depletion. Iodoacetate, for example, has a high reactivity with sulfydryl groups of other proteins, in addition to glycolysis inhibiting properties (Webb, 1966). Moreover, the presence of oxygen during chemical anoxia can lead to the production of reactive oxygen metabolites which themselves have cell injuring properties (Gores et al., 1989a; Dawson et al., 1993).

Hypoxia is another model for investigating ischemia-induced injuries. This model requires the reduction of the oxygen pressure below the critical level of complete oxydation of cytochrome C (Weinberg, 1991). As a result, mitochondrial respiration will be inhibited or abolished leading to ATP depletion. Similar to the chemical anoxia model, hypoxia involves injury induced by reactive oxygen metabolites since oxygen is still present.

Finally, anoxic conditions have been used. In contrast to both chemical anoxia and hypoxia, in this situation oxygen is completely absent and production of reactive oxygen metabolites is precluded. The exclusion of reactive oxygen metabolites was the major reason to choose the anoxic model in the present study. In addition, the hypoxic model refers to any oxygen level below normoxia which means that dependent on the level obtained, different injuries will develop since different cellular processes may have different oxygen dependencies. In anoxic studies such differences are prevented.

Outline of the study

The present study will focus on the role of $[Ca^{2+}]_i$ in anoxia-induced cell injury in renal epithelial cells. A prominent role of Ca^{2+} in ischemia-induced injury was first suggested in the isolated perfused kidney model in which Ca^{2+} entry blockers were shown to have a protective effect (Burke et al., 1984; Schrier, 1987). Nevertheless, these studies did not reveal whether the protective effects of Ca^{2+} channel blockers are on vascular or tubular cells. Moreover, the existence of L-type Ca^{2+} channels in renal epithelial cells was not demonstrated so far. In this respect we conducted binding studies with Ca^{2+} channel blockers, which revealed significant binding of the phenylalkylamine desmethoxy-verapamil to proximal tubule basolateral membranes (Bindels et al., unpublished

data), indicating the presence of L-type Ca^{2+} channels. To clarify whether Ca^{2+} channel blockers blocked these channels and whether they had a protecting effect on tubular cells during anoxia, experiments on isolated tubular cells were performed.

In chapter 2, an *in vitro* model was developed to measure $[Ca^{2+}]_i$ in cultured PT cells during substrate free anoxia. With this model, the relation between changes in $[Ca^{2+}]_i$ and the development of cell injury was investigated. Especially the involvement of Ca^{2+} influx via L-type Ca^{2+} channels was studied with the use of Ca^{2+} channel blockers.

In chapter 3, anoxic experiments were performed with freshly-isolated PT cells. Anoxia-induced increases in $[Ca^{2+}]_i$ were measured and the effects of Ca^{2+} channel blockers studied. In addition to the increases in $[Ca^{2+}]_i$, cell viability was determined after 1 h of anoxia, and the correlation between elevated $[Ca^{2+}]_i$ and loss of cell viability was studied.

In chapter 4, pH_i measurements in both cultured and freshly-isolated PT cells during anoxia are described.

In chapter 5, the anoxic model used in the previous chapters was compared to a chemical anoxia model in which anoxia is mimicked by incubation of cells with metabolic inhibitors, in the presence of oxygen.

Since *in vivo* studies already demonstrated that ischemia does not result in homogeneous injury in the different cells in the kidney, we investigated the effect of anoxia in mTAL and cTAL cells in chapter 6.

Finally, the effect of anoxia on $[Ca^{2+}]_i$ was studied in isolated ventricular cardiomyocytes in chapter 7. The reason for studying the responses to anoxia of cardiomyocytes was, that anoxic $[Ca^{2+}]_i$ in renal epithelial cells was relatively modest and the literature indicated that $[Ca^{2+}]_i$ is much higher in anoxic excitable cells.

CHAPTER 2

The effect of L-type Ca^{2+} channel blockers on anoxia-induced increases in intracellular Ca^{2+} concentration in rabbit proximal tubule cells in primary culture

U.M. Rose, R.J.M. Bindels, A. Vis, J.W.C.M. Jansen and C.H. Van Os
in: Pflügers Archiv 423: 378-386 (1993)

ABSTRACT

Ca^{2+} channel blockers have been shown to be protective against ischemic damage of the kidney, suggesting an important role for $[Ca^{2+}]_i$ in generating cell damage. To delineate the mechanisms behind this protective effect, we studied $[Ca^{2+}]_i$ in cultured PT cells during anoxia in the absence of glycolysis and the effect of metyhoxyverapamil (D600) and felodipine on $[Ca^{2+}]_i$ during anoxia. A method was developed whereby $[Ca^{2+}]_i$ in cultured PT cells could be measured continuously with a fura-2 imaging technique during anoxic periods up to 60 min. Complete absence of O_2 was realized by inclusion of a mixture of oxygenases in an anoxic chamber. $[Ca^{2+}]_i$ in PT cells started to rise after 10 min of anoxia and reached maximal levels at 30 min, which remained stable up to 60 min. The onset of this increase and the maximal levels reached varied markedly among individual cells. The mean values for normoxic and anoxic $[Ca^{2+}]_i$ were 118 ± 2 (N = 98) and 662 ± 22 (N = 160) nM, respectively. D600 (1 μM), but not felodipine (10 μM), significantly reduced basal $[Ca^{2+}]_i$ in normoxic incubations. During anoxia, 1 μM and 100 μM D600 significantly decreased anoxic $[Ca^{2+}]_i$ levels by 22 and 63%, respectively. Felodipine at 10 μM was as effective as 1 μM D600. Removal of $Ca^{2+}{}_o$ and addition of 0.1 mM La^{3+} completely abolished the anoxia-induced increases in $[Ca^{2+}]_i$. We conclude that anoxia induces increases in $[Ca^{2+}]_i$ in rabbit PT cells in primary culture, which results from Ca^{2+} influx. Since this Ca^{2+} influx is partially inhibited by low doses of Ca^{2+} channel blockers, L-type Ca^{2+} channels may be involved.

INTRODUCTION

Investigations into the pathophysiology of ischemic renal cell injury have revealed that L-type Ca^{2+} channel blockers can provide protection under various conditions (Schwertschlag et al., 1986; Schrier, 1987; Shimizu et al., 1990; Silverman et al., 1990; Talén et al., 1991). Burke et al. (1984) demonstrated that verapamil, a phenylalkylamine, protected renal function and decreased morphological injury, as well as the Ca^{2+} content of renal tissue, during an *in vivo* ischemic period. Verapamil also improved renal function after warm or cold ischemia in isolated perfused rat kidneys (Silverman et al., 1990). In addition, in humans, Ca^{2+} channel blockers reduce the incidence of post-transplant acute tubular necrosis (Wagner et al., 1987). Taken together, these studies have suggested a role for increased Ca^{2+} influx through voltage-dependent Ca^{2+} channels in the pathogenesis of ischemic renal cell injury. However, these studies do not clearly document whether the protective effect of Ca^{2+} channel blockers is primarily on vascular or epithelial cells. In addition, L-type Ca^{2+} channels have not been demonstrated unequivocally in renal tubular cells (Weinberg, 1991). Evidence for the presence of such channels in the proximal tubule originates from recent studies on cell volume regulation (McCarty et al., 1991a, 1991b). McCarty and O'Neil (1991b) showed that $[Ca^{2+}]_i$ in PT cells was significantly reduced by 10 μM verapamil but not nifedipine, a dihydropyridine. During cell swelling, however, also nifedipine-sensitive Ca^{2+} channels became activated (McCarty et al., 1991b). Another recent study demonstrated that Ca^{2+} channel blockers decreased uptake of radiolabelled Ca^{2+} in freshly-isolated PTs exposed to anoxia suggesting Ca^{2+} influx via L-type Ca^{2+} channels (Almeida et al., 1992). So far, only few studies have used Ca^{2+} sensitive fluorescent probes to directly measure changes in $[Ca^{2+}]_i$ after exposure to hypoxia or chemical anoxia (Smith et al., 1992; Jacobs et al., 1991), and no studies are known in which effects of Ca^{2+} channel blockers have been studied directly on anoxia-induced increases in $[Ca^{2+}]_i$.

In the present study, we developed a method to investigate whether anoxia induces an increase in $[Ca^{2+}]_i$ in PT cells. As a model system, primary cultures of rabbit PT cells were chosen whereby $[Ca^{2+}]_i$ can be measured conveniently with a fura-2 imaging technique during anoxic periods of 45 to 60 min. Complete absence of O_2 was realised by inclusion of a novel mixture of

oxygenases (Oxyrase®; Oxyrase Inc., Ashland, Ohio, USA) during cell incubation in an anoxic chamber. Such an anoxia model precludes any contribution of oxygen free radicals to cellular injury, which may occur in hypoxia and chemical anoxia (Weinberg, 1991). Finally, we studied the effect of Ca^{2+} channel blockers on anoxia-induced increases in $[Ca^{2+}]_i$ to investigate whether protective effects of these agents can be anticipated from a reduction in $[Ca^{2+}]_i$ during ischemic insults.

MATERIALS AND METHODS

Primary culture of proximal tubules

Rabbit kidney PT cells were isolated by immunodissection and subsequently cultured as described previously (Bindels et al., 1991). Briefly, kidneys were excised from New Zealand white rabbits (≈0.5 kg). A cortical cell suspension, obtained by enzymatic digestion of dissected cortical tissue, was incubated for 60 min on ice with monoclonal antibodies (mAb) 85C8 and 101E12, recognizing cell surface antigens specific for the proximal tubule. After three washings, the cell suspension was added to goat anti-mouse IgG-coated petri dishes and incubated for 15 min at 20 °C. The dishes were washed carefully and adherent PT cells were scraped off the dishes. The immunodissected PT cells were seeded on collagen-coated round coverslips (Ø 22 mm; Menzel, Germany) or on collagen-coated 96-well plates (Costar, Badhoevedorp, The Netherlands) at a density of $2x10^5$ cells/cm^2. Cells were grown to confluency in a mixture of Dulbecco's Modified Eagles medium (Imperial #1-466-14, Hampshire, UK): Ham's F12 medium (Gibco, #041-01765 M, Paisley, UK) (1:1), supplemented with gentamycin (10 µg/ml), $NaHCO_3$ (25 mM), glutamine (14 mM), insulin (5 µg/ml), transferrin (5 µg/ml), hydrocortisone (50 nM), 0.5% (v/v) non-essential amino acids (Gibco, #043-01140 H, Paisley, UK), prostaglandin E_1 (70 ng/ml), triiodothyronine (5 pM) and Na_2SeO_3 (50 nM), pH 7.4; hereafter this culture medium is referred to as K_1 medium. During the first 24 h of culture 5% (v/v) FCS was present in the K_1 medium. For all experiments, PT cells were used 5 to 6 days after seeding.

Characterization of the primary culture of proximal tubule cells

Several methods were used to characterize the primary culture of PT cells.

1. PT cells were characterized by conventional immunoperoxidase staining (Brown et al., 1971) with mAb 85C8 and 101E12.

2. Hormone-induced cAMP formation was measured to further characterize the PT primary culture. PT cells cultured on 96-well plates were washed twice with Krebs-Henseleit buffer (KHB; composition in mM: 128 NaCl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 10 Na-acetate, 4 l-lactate, 1 l-alanine, 20 HEPES/Tris, pH 7.4), and incubated at 37 °C with 100 µl/well 10^{-7} M

bPTH(1-34), 10^{-7} M isoproterenol or 10^{-7} M calcitonin in KHB containing 1 mM IBMX. After 10 min, the reaction was stopped by aspiration of the buffer and addition of 50 µl 0.2 N HCl to each well. The cAMP concentration was determined according to Brown et al (1971)

3. Hormone-induced $[Ca^{2+}]_i$ signals were investigated in PT cells cultured on glass coverslips and loaded with fura-2, as described in the next section. The effects of 5 µM bradykinin and 5 µM angiotensin II were tested

Fura-2 loading of PT cells in primary culture

PT cells were loaded with fura-2 by incubating coverslips with PT cell monolayers for 1 h at 37 °C in K_1 medium containing 5 µM fura-2 AM (Molecular Probes, Eugene, OR, USA), 0.02% (w/v) pluronic F127 (Molecular Probes, Eugene, OR, USA), 4% (v/v) FCS and 3 mM probenecid (Sigma, St. Louis, MO, USA). After loading, the cells were washed twice in the experimental medium and were used immediately All experiments were performed in the presence of 3 mM probenecid in order to inhibit fura-2 leakage via anion exchangers

Measuring $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by quantitative fluorescence microscopy using two systems. Firstly, the Newcastle Photonic system (NPS, Newcastle, UK) uses a photomultiplier tube which is connected to a Nikon Diaphot inverted microscope with a 40x quartz oil immersion objective to monitor single PT cells The photometer contains a pin-hole diaphragm to regulate the viewing field. The data in our study represent fluorescence measurements from 2 to 4 cells, captured at 400x magnification. The fura-2 loaded PT cells were alternatingly excited at 340 and 380 nm and emitted light was collected at 1-s intervals at 510 nm. Secondly, in some studies the MagiCal system (Joyce Loeble, Tyne & Wear, UK) was used in which emitted light is captured with a CCD camera followed by digital imaging using TARDIS® software (Joyce Loeble, Tyne & Wear, UK) The 340-380 nm capturing sequence was interrupted by 30 s of no capturing, divided into 10 s excitation at 380 nm allowing for cell focusing, and 20 s of no excitation using a shutter to avoid bleaching. The MagiCal system has been described in detail by Neylon et al.(1990)

$[Ca^{2+}]_i$ was calculated according to the formula derived by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_D \times R_{bf} \times [(R-R_{min}) / (R_{max}-R)]$, where K_D is the dissociation constant of fura-2 for Ca^{2+} of 224 nM, R is the ratio of fluorescence of the cell at 340 and 380 nm, R_{max} and R_{min} represent the ratios of fura-2 fluorescence intensity at 340 and 380 nm excitation obtained by treating the monolayers with 5 µM ionomycin in the presence and absence of Ca^{2+}_o, respectively; R_{bf} is the maximal 380 nm signal divided by the minimal 380 nm signal. The fluorescence was corrected for background fluorescence estimated in the presence of 1 mM $MnCl_2$ A calibration procedure could not be performed on every cell or preparation due to technical limitations. Firstly, addition of the ionophore ionomycin induced abrupt rounding off of the PT cells leading to cell detachment from the coverslip, resulting in focusing

problems. Secondly, the addition of EGTA, to ionomycin-containing solutions, frequently resulted in detachment of the few cells still attached. Most results are therefore presented as ratio values instead of real Ca^{2+} concentrations. In figure 9 and in Table II, Ca^{2+} concentrations are also presented, which are based on a few successful calibration procedures.

Anoxic chamber experiment

In order to estimate $[Ca^{2+}]_i$ during anoxia, a coverslip with fura-2-loaded PT cells was placed in an anoxic chamber. This chamber was a modified version of the microperfusion chamber described by Ince et al. (1990) and contained the following elements: an inlet and outlet for incubation medium, a temperature sensor and a coiled stainless tube through which warm water can be passed to obtain an internal temperature of 37 °C. Experiments were started by filling the chamber with 5 ml KHB with or without glucose (KHB ± glucose), previously gassed with 100% N_2. In addition, KHB ± glucose was supplemented with the oxygen reducing enzyme Oxyrase® (360 mU/ml) (Joseph et al., 1990). After filling, the chamber was placed on the microscope stage of the fluorescence measuring system and $[Ca^{2+}]_i$ was measured. Simultaneous with the measurement of $[Ca^{2+}]_i$, partial O_2 pressure (PO_2) was monitored using a Clark-type oxygen electrode. The electrode consisted of a platinum cathode and silver anode connected through an electrolyte solution (1 M KCl : glycerol as 1: 1) and covered with a 6 µm Teflon membrane. The electrode was calibrated in KHB gassed with 100% N_2 (= 0% O_2) or air (= 20% O_2) at 37 °C.

Cell viability and LDH release

PT monolayers on coverslips were mounted in the anoxic chamber at 37 °C containing the incubation medium as described above. After 60 min of anoxia, medium and cells were collected separately for LDH activity measurements. Herefore, cells were scraped off the coverslip in 1 ml MilliQ® (Millipore) water and the resultant cell suspension was sonicated for 30 s at 100 Watt to release the LDH. Both cell and medium samples were centrifuged for 5 min at 200 x g and LDH content was measured by incubating a sample of the supernatant in 0.6 mM pyruvate and 0.18 mM NADH. The decrease in NADH concentration, a marker for LDH activity, was monitored at 340 nm in a DW 2000 spectrophotometer (SLM Instruments, Urbana, Ill, USA). All results are presented as % LDH of total LDH, which is the sum of LDH activity in the cells and medium. Oxyrase® incidently also contained LDH activity which is 20 to 30% of total LDH present in the cells and medium. In addition, trypan blue exclusion was estimated by incubating the monolayer in 0.08% (w/v) trypan blue. The percentage stained cells was determined by counting under light microscopy. Some monolayers were also incubated in 1 µg/ml ethidium bromide. In this case, stained cells were counted under UV light microscopy. By way of control, several monolayers were experimented in the presence of oxygen.

Materials

The hormones bPTH (1-34), isoproterenol, calcitonin, bradykinin, angiotensin II and conjugated antibodies were obtained from Sigma (St. Louis, MO, USA). The Ca^{2+} channel blockers D600 (methoxyverapamil) and felodipine were kindly provided by Knoll AG (Ludwigshafen, Germany) and Astra Pharmaceutica (Rijswijk, The Netherlands), respectively. All chemicals were of the purest grade.

Statistical analysis

All reported data are expressed as means ± SE. Statistical analysis was performed on ratio values using analysis of variance ($P < 0.05$ is significant). Subsequently, statistical significant differences between experimental groups were estimated by means of contrast analysis according to Fisher (Snedecor et al., 1974).

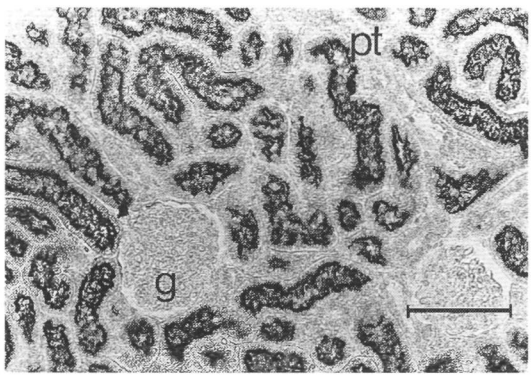


Figure 1
Immunoperoxidase staining with monoclonal antibodies 85C8 and 101E12 of paraffin-embedded sections of rabbit kidney. Only proximal tubules are positively stained. Glomerulus (g) and proximal tubule (pt) are indicated. The bar represents 100 μm.

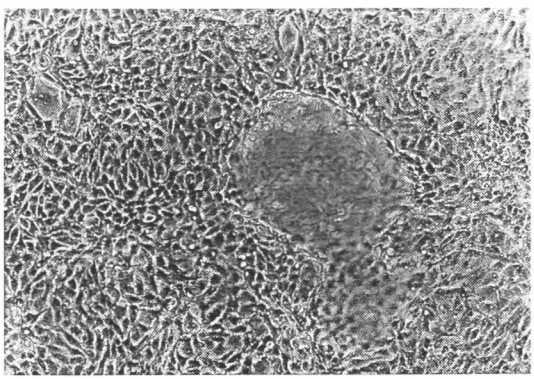


Figure 2
Phase contrast micrograph of a 6 day-old primary culture of rabbit proximal tubules on collagen-coated glass coverslips. Monolayers exhibit an epithelial-like appearance and dome formation. Original magnification x100.

RESULTS

Characterization of the PT cell culture

Conventional immunoperoxidase staining with mAb 85C8 and 101E12 confirmed that these antibodies specifically recognize PTs (Fig.1). mAb 85C8 and 101E12 did not react with glomeruli, loops of Henle, distal convoluted tubules, connecting tubules or collecting ducts. The immunodissected PT cells were cultured and reached confluency with an epithelial-like appearance within 4 days of seeding. The monolayers formed "domes" after 5 to 6 days (Fig. 2), which is indicative of transepithelial NaCl and H_2O transport (Toutain et al., 1991).

Table I. Hormone-induced cAMP formation in rabbit proximal tubules in primary culture

Hormone	cAMP (nmol.mg protein $^{-1}$ h $^{-1}$)
Control	306 ± 72
bPTH (1-34)	4098 ± 438*
isoproterenol	2790 ± 426*
calcitonin	714 ± 150*

Values are means ± SE of 10 experiments. In all experiments, hormones were applied at a concentration of 10^{-7} M. *P < 0.05, significantly different from control.

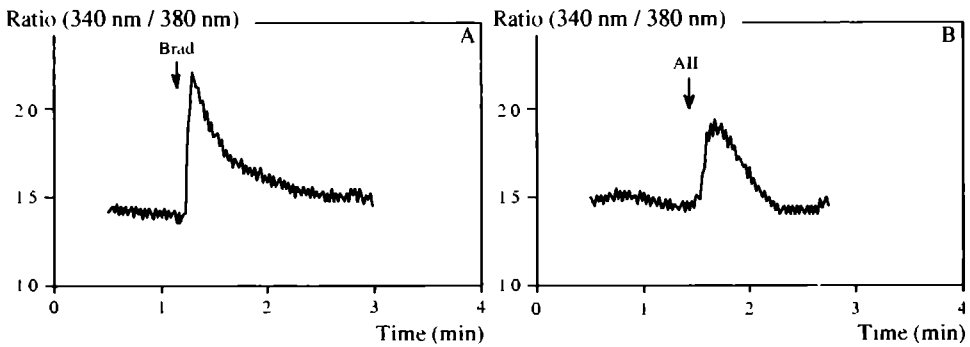


Figure 3

Addition of 5 µM bradykinin (A, brad) or 5 µM angiotensin II (B, AII) to fura-2 loaded proximal tubule monolayers. $[Ca^{2+}]_i$ is presented as the ratio of 340 and 380 nm fluorescence signals of 2 to 4 cells.

Table I shows the effects of hormones on intracellular cAMP levels of cultured PT cells. PTH as well as the ß-adrenergic agonist isoproterenol stimulated cAMP production by 1 order of magnitude. The presence of receptors for both substances on PT cells is well established (Morel, 1981). Although calcitonin receptors are absent from PT cells, in the cultured PT cells calcitonin stimulated cAMP production, but the effect of calcitonin was only one-fifth of the PTH effect. In addition to hormone-induced cAMP formation, the effect of hormones on $[Ca^{2+}]_i$ in cultured PT cells was investigated. A transient $[Ca^{2+}]_i$ increase was found after addition of bradykinin or angiotensin II (Figs. 3A and B).

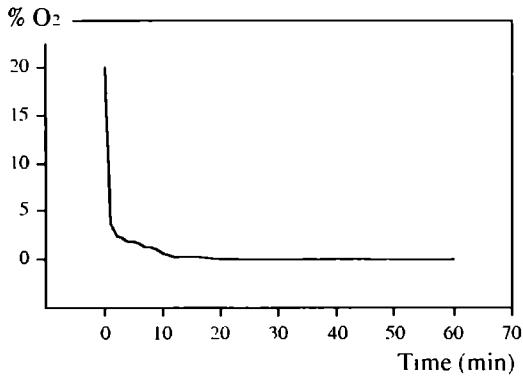


Figure 4
Oxygen pressure (PO_2) in the anoxic chamber: a rapid PO_2 decline as a result of filling the anoxic chamber with hypoxic medium is followed by a gradual reduction of O_2 by the enzyme Oxyrase. For PO_2 measurements, a calibrated O_2 electrode was positioned in the anoxic chamber. PO_2 is presented as $\%O_2$.

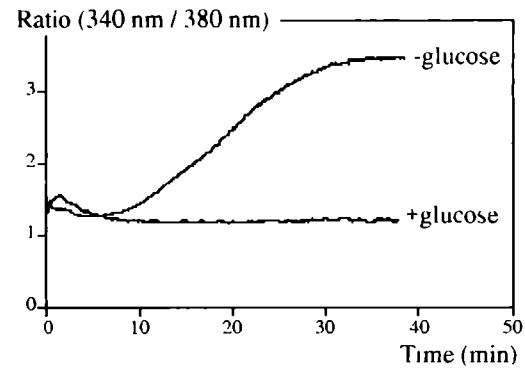


Figure 5
Typical measurements of $[Ca^{2+}]_i$ with quantitative fluorescence microscopy (the photomultiplier system) during anoxic incubation of PT cells on a glass coverslip in the presence (+glucose) or absence (-glucose) of glucose. $[Ca^{2+}]_i$ is presented as 340 and 380 nm ratios of 2-4 cells.

$[Ca^{2+}]_i$ during anoxia

PO_2 in the anoxic chamber during Ca^{2+} measurements was estimated using an oxygen electrode inserted into the chamber. Figure 4 shows the PO_2 as a function of time. In the first few minutes, PO_2 decreased rapidly as a result of filling the chamber with hypoxic medium. After this rapid decline, the residual oxygen was reduced slowly by the enzyme complex Oxyrase®, resulting in total elimination of oxygen. Figure 5 shows that $[Ca^{2+}]_i$, measured during anoxia in

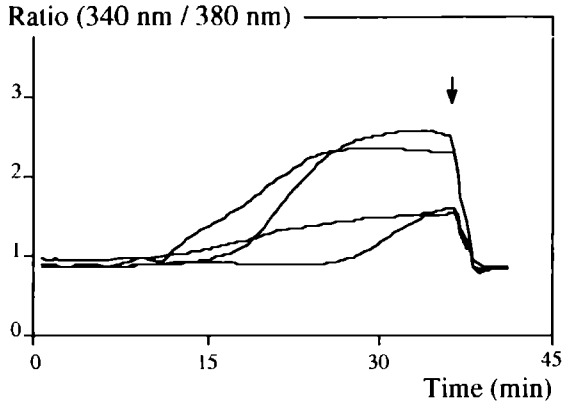


Figure 6
Individual $[Ca^{2+}]_i$ traces of four PT cells responding to O_2 and glucose deprivation at 37 °C. After 10 min of anoxia, $[Ca^{2+}]_i$ increased, reaching a maximal level after 30 to 40 min. At the introduction of oxygen and glucose (arrow), $[Ca^{2+}]_i$ returned to basal levels. $[Ca^{2+}]_i$, presented as ratio values, was measured on the MagiCal system.

KHB + glucose did not change for up to 1 h of anoxia. An explanation for the stable $[Ca^{2+}]_i$ could be the presence of glucose and acetate, which provides sufficient ATP through the glycolytic pathway (Hugo-Wisseman et al., 1991). The anoxic experiment was, therefore, repeated with KHB excluding glucose and acetate as glycolytic substrates. As a result of anoxia and substrate exclusion, $[Ca^{2+}]_i$ started to rise after 10 min of anoxia and reached a maximal level within 30 min (Fig.5), which was maintained during the subsequent 30 min anoxic period. All further studies were carried out in the absence of glucose and acetate. These results were obtained from groups of cells using the photomultiplier set-up. However, in order to determine $[Ca^{2+}]_i$ during anoxia in several individual cells at the same time, the MagiCal imaging system was used. Again, anoxia led to an increase in $[Ca^{2+}]_i$ and the reintroduction of oxygen and substrates resulted in the return of elevated $[Ca^{2+}]_i$ levels to basal or even subbasal values (Fig.6, Table II). The onset of this rise and the maximal levels reached varied strikingly among individual cells. This heterogeneity was not only observed among PT cells on the same coverslip, but also between cells from different preparations (Fig.7). In Table II the mean values are given for all fura-2 ratios presented in Fig. 7 with the corresponding $[Ca^{2+}]_i$ values. The data show that upon reperfusion after 60 min of anoxia, $[Ca^{2+}]_i$ levels are significantly lower than at the onset of anoxia.

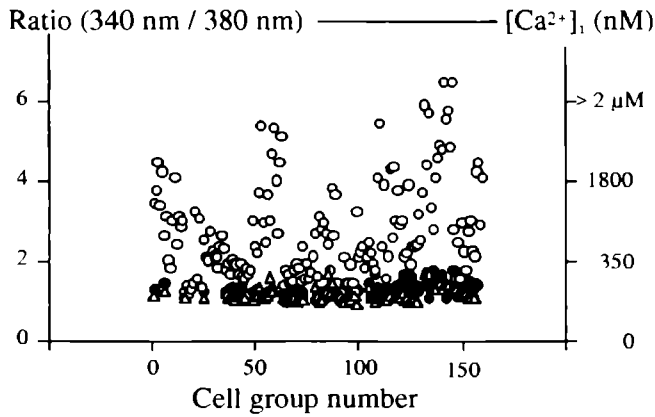


Figure 7

Heterogeneous responses of PT cells obtained from several isolations to anoxic incubation: •, basal $[Ca^{2+}]_i$ measured immediately after filling the anoxic chamber: o, maximal $[Ca^{2+}]_i$ reached at 30 min of anoxia; Δ, $[Ca^{2+}]_i$ reached after reperfusion. Each point represents the ratio or $[Ca^{2+}]_i$ value observed in 2 to 4 cells using the photomultiplier system.

Table II. Anoxia-induced increase in $[Ca^{2+}]_i$

Condition	Parameter	
	340/380 nm ratio	$[Ca^{2+}]_i$ (nM)
Basal	1.30 ± 0.02	118 ± 2
Anoxia	2.73 ± 0.09*	662 ± 22*
Reperfusion	1.14 ± 0.02*	76 ± 1*

Mean values ± SE of 340/380 nm ratios and $[Ca^{2+}]_i$ of the data presented in Fig 7. *Significantly different from basal value. Cultured PT cells were incubated under anoxic conditions for 60 min after which period the chamber was reoxygenated.

Since verapamil has been shown to reduce ischemic renal injury when present during the ischemic insult (Humes, 1986; Cotterill et al., 1989), the possibility of whether D600 could reduce the rise in $[Ca^{2+}]_i$ during anoxia was tested. In figure 8A it is shown that the presence of 1 µM D600 decreased $[Ca^{2+}]_i$ significantly. Firstly, 1 µM D600 reduced the basal fluorescence ratio from 1.50 ± 0.02 to 1.42 ± 0.02 ($P < 0.05$), and secondly, the maximal ratio reached during anoxia was diminished from 2.28 ± 0.05 to 2.03 ± 0.06 ($P < 0.05$). In addition, D600 reduced the difference between basal and maximal $[Ca^{2+}]_i$ ratio values in control and D600 experiments from 0.77 ± 0.05 to 0.60 ± 0.05, respectively ($P < 0.05$). After reperfusion, no difference in $[Ca^{2+}]_i$ between

D600 and control experiments was found. Additionally, the effects of 100 μm D600 and of 1 and 10 μM felodipine on the anoxia-induced increase in $[Ca^{2+}]_i$ were studied (Table III). Felodipine at 10 μM significantly reduced $[Ca^{2+}]_i$ during anoxia ($P < 0.05$), but at 1 μM the effect was smaller than the effect of 1 μM D600. However, felodipine at 10 μM was as effective as 1 μM D600. Surprisingly, an increase in the concentration of D600 from 1 to 100 μM further reduced $[Ca^{2+}]_i$ during anoxia (Table III).

Table III. Effects of Ca^{2+} channel blockers on anoxia-induced increases in $[Ca^{2+}]_i$

Parameter	Condition			
	Control	D600 (1 μM)	Control	D600 (100 μM)
Basal (Ratio)	1.50 ± 0.02	1.42 ± 0.02*	1.25 ± 0.08	1.05 ± 0.02*
Anoxic (Ratio)	2.28 ± 0.05	2.03 ± 0.06*	2.50 ± 0.15	1.60 ± 0.06*
Anoxic $[Ca^{2+}]_i$ (nM)	448 ± 10	348 ± 9*	547 ± 32	203 ± 8*
	Control	Felodipine (1 μM)	Control	Felodipine (10 μM)
Basal (Ratio)	1.36 ± 0.03	1.38 ± 0.02	1.36 ± 0.02	1.36 ± 0.01
Anoxic (Ratio)	2.75 ± 0.08	2.46 ± 0.08*	2.42 ± 0.07	1.99 ± 0.07*
Anoxic $[Ca^{2+}]_i$ (nM)	673 ± 19	527 ± 18*	508 ± 15	335 ± 12*

Anoxic incubation of PT cell monolayers in the presence of D600 (1 μM or 100 μM) or felodipine (1 or 10 μM), or in the absence of Ca^{2+} channel blockers (control). Each experimental condition has its own control. Basal and anoxic $[Ca^{2+}]_i$ is presented as 340/380 nm ratios, $[Ca^{2+}]_i$ after 45 min of anoxia is also given in nM. All data are the mean ± SE of at least 50 cell groups (*$P < 0.05$, significantly different from control).

Next, the dependence of the anoxia-induced increase in $[Ca^{2+}]_i$ on Ca^{2+}_o was studied. A nominally Ca^{2+} free solution (≈20 μM $[Ca^{2+}]_o$) partially prevented the anoxia-induced increase in $[Ca^{2+}]_i$ (Fig. 8B). Further reduction in Ca^{2+}_o by adding EGTA was not feasible since the cells detached from the coverslip. However, addition of 0.1 mM La^{3+} to the nominally free Ca^{2+} solution resulted in total abolishment of anoxia-induced increases in $[Ca^{2+}]_i$ (Fig. 8C).

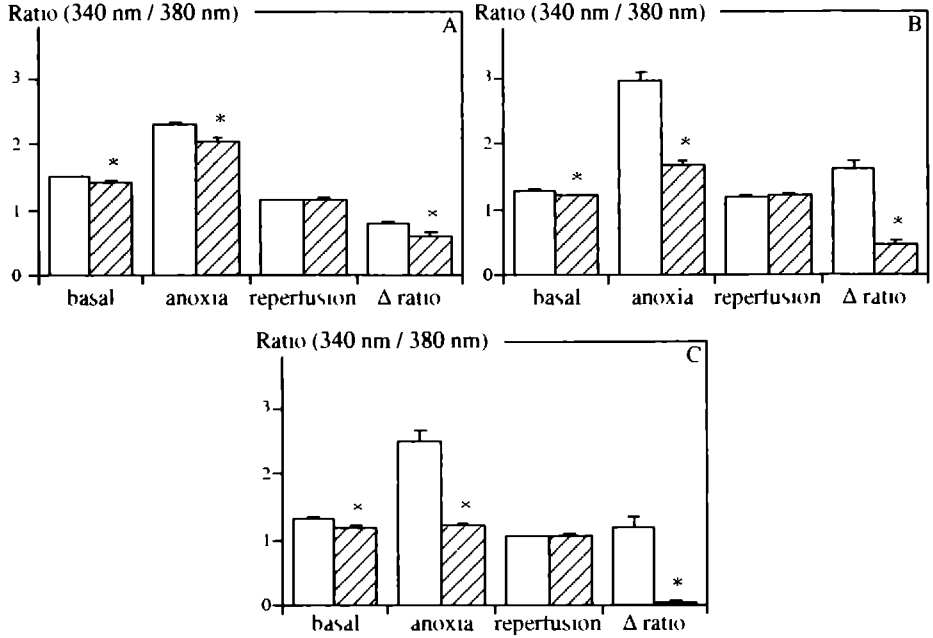


Figure 8

The effect of 1 μM D600 (**A**), extracellular Ca^{2+} exclusion (**B**) and the combination of extracellular Ca^{2+} exclusion and 0.1 mM $LaCl_3$ (**C**), on basal, maximal anoxic and reperfusion ratio values, and on Δratio values (maximal minus basal ratios). Experimental ratios (dashed columns) are compared to control ratios (open columns), i.e. anoxic incubation in the presence of 2 mM $CaCl_2$. Columns represent mean ratio values ± SE with N is ≥ 22 (*P < 0.05: experimental versus control ratio values).

LDH release and cell injury

In addition to the increases in $[Ca^{2+}]_i$ induced by anoxia and substrate deprivation, the effects on cell viability were tested using trypan blue and ethidium bromide staining. No change in cell staining could be detected as a result of 60 min of anoxia and substrate exclusion. Moreover, no significant fura-2 leakage occurred during anoxia (345,853 ± 29,760 fluorescent counts at the beginning of anoxia versus 317,798 ± 24,133 counts after 60 min of anoxia; $P > 0.2$); indicative of no change in viability throughout the 60 min period. When the LDH content of the incubation medium was corrected for endogenous LDH from Oxyrase®, there was no significant release of LDH above the control values of 4 ± 1% in the oxygenated PT cells.

DISCUSSION

In the present study, an *in vitro* model consisting of primary cultures of rabbit PT cells was developed to study changes in $[Ca^{2+}]_i$ in single cells during prolonged periods of anoxia. The response of individual cells to anoxia was remarkably heterogeneous, both with respect to the onset of increases in $[Ca^{2+}]_i$ and to the steady-state level reached after 30 to 40 min of anoxia. Phenylalkylamines and, less efficiently, dihydropyridines partly reduced anoxia-induced increase in $[Ca^{2+}]_i$ during anoxia.

The primary culture, in contrast to previous studies (Schwertschlag et al., 1986; Thevenod et al., 1986), originates from immunodissected PT cells using mAb directed against a brush border antigen. In this way, only PT cells are isolated and can be seeded at high density. Previously, we demonstrated that this strategy yielded confluent monolayers of cortical collecting tubule cells which retained characteristic functions of the original nephron segment (Bindels et al., 1991). In the present study, confluent monolayers of PT cells were obtained within 4 days after seeding. The cultured PT cells expressed functional receptors for PTH, the ß-adrenergic agonist isoproterenol, bradykinin and angiotensin II, of which the first two are coupled to adenylate cyclase and the last two to phospholipase C. The presence of these receptors has been previously demonstrated on freshly-isolated PT cells (Morel, 1981; Thevenod et al., 1986; Goligorsky et al., 1988; Talén et al., 1991).

Cytosolic Ca^{2+} in PT cells cultured on coverslips could be conveniently studied in an anoxic chamber on an inverted microscope. Fura-2 fluorescence was monitored up to 60 min without difficulty since bleaching or dye leakage were insignificant with a near density filter of 3.0 in the excitation pathway and 3 mM probenecid in the incubation medium. In order to study potential protective effects of Ca^{2+} channel blockers in an anoxic model, conditions should tender unambiguous interpretation of the results. In hypoxia, as well as in chemical anoxia models, superoxide radicals can be formed, possibly resulting in lipid peroxidation and destruction of membrane barrier properties (Turrens et al., 1985; Herman et al., 1988). Therefore, complete anoxia, i.e. PO_2=0 mmHg, was established in our model system by adding an oxygenase mixture to the medium. Total elimination of O_2 from the media has always been troublesome, even when very high gassing rates were applied (Jacobs et al., 1991; Almeida et

al., 1992). Jacobs et al. (1991) were able to reach zero PO_2 in a very dense PT suspension (3 mg protein/ml) in which residual O_2 was rapidly consumed by PT cells. In the present study, only 0.7 mg protein/ml was present in the chamber, but zero PO_2 is reached within 10 min due to the presence of Oxyrase®. One disadvantage of Oxyrase®, however, is its contamination of LDH which amounts to approximately 25% of the total LDH present in PT cells on one coverslip. This interferes seriously with the detection of the onset of cell death. For example, in our chamber LDH release in 60 min control incubations ranges between 2 and 5% of total LDH. During anoxia, an extra amount of 25% of total LDH is present in the chamber as a contaminant from Oxyrase®, which obscures the first 5% of anoxia-induced LDH release in these experiments. However, limited injury was unlikely, since trypan blue exclusion and ethidium bromide staining were not altered after 60 min of anoxia.

During anoxia, $[Ca^{2+}]_i$ increased only in the absence of glucose and acetate, indicative of otherwise sufficient ATP supplied by glycolytic pathways. This is in line with previous studies which showed that primary cultures of PT cells tend to be more glycolytically active and more resistant to O_2 deprivation (Snowdowne et al., 1985; Tang et al., 1989). In the absence of O_2 and substrates for glycolysis, we observed increases in $[Ca^{2+}]_i$ in all cells studied. The onset of rise varied between 5 and 20 min after PO_2 was reduced to zero. This result suggests that ATP depletion occurs in all cells. $[Ca^{2+}]_i$ reached maximal levels within 30 min after the initial increase and this level, albeit highly variable among individual cells, remained stable until O_2 was reintroduced. This phenomenon strongly suggests some kind of endogenous autoprotective mechanism, whereby the ATP-depleted state decreases Ca^{2+} permeability of the plasma membrane. An intriguing possibility is that intracellular acidosis contributes to this phenomenon, which has been shown in freshly-isolated PTs (Burnier et al., 1988). Whether intracellular acidosis occurs and whether it contributes to reducing Ca^{2+} influx in cultured PT cells during anoxia remains to be established.

Our observation that the increase in $[Ca^{2+}]_i$ was completely abolished by removing Ca^{2+}_o and adding La^{3+} is in line with a recent study which reports effects of chemical anoxia on $[Ca^{2+}]_i$ in cultured PT cells (Smith et al., 1992). These observations suggest that intracellular Ca^{2+} stores do not contribute to Ca^{2+} overload during anoxic periods in our model. A possible explanation could

be that when intracellular Ca^{2+} stores begin to release Ca^{2+}, the plasma membrane Ca^{2+}-ATPase may still operate at low ATP concentrations. In addition, an early increase in $[Ca^{2+}]_i$ would activate calmodulin and thereby stimulate the plasma membrane Ca^{2+}-ATPase. It should be brought to mind that most of our measurements were made with the photomultiplier system which is unable to monitor spatial differences in $[Ca^{2+}]_i$. The imaging system used in some preparations is also not sensitive enough to measure a heterogenous distribution of Ca^{2+} in the cytosol. Jacobs et al. (1991) reported no substantial increase in $[Ca^{2+}]_i$ in freshly-isolated PTs during anoxia. This discrepancy with our study may be related with the different preparations used. In two other studies using cultured PT cells, modest increases in $[Ca^{2+}]_i$ were seen after inhibition of metabolism (McCoy et al., 1988; Phelps et al., 1989). When all our observations in single cells are averaged, the resulting increase in $[Ca^{2+}]_i$ produced by anoxia is relatively modest (Fig. 7, Table II). Only a small percentage of cells exhibit increases in $[Ca^{2+}]_i$ above 1 μM and even these cells do not leak fura-2 as a sign of cell death. Contrarily, when lethal oxidative stress was imposed on cultured hepatocytes, an abrupt and much larger rise in $[Ca^{2+}]_i$ preceded cell death, which was monitored as loss of fura-2-fluorescence (Sakaida et al., 1991). This phenomenon was never observed in our study which agrees well with the absence of LDH leakage from the cells. Another indication that primary cultures of PT cells survive 60 min of anoxia is the observation that in all cells $[Ca^{2+}]_i$ returns to control levels within a few min after the reintroduction of oxygen. Although the long-term effects of reperfusion on cell viability have not been studied, our PT cell monolayer system offers a feasible model. As yet there is little information on the behaviour of $[Ca^{2+}]_i$ during conditions analogous to *in vivo* reperfusion-induced cell injury (Wagner et al., 1987).

The present study provides functional evidence for the presence of L-type Ca^{2+} channels in PT cells in primary culture. D600, at 1 μM, reduced basal $[Ca^{2+}]_i$ levels significantly (Table III). Only one other study using isolated PTs of the rabbit reported that 10 μM verapamil, but not felodipine, reduced basal $[Ca^{2+}]_i$ (McCarty et al., 1991b). Similarly, we demonstrated that felodipine was without effect, confirming the notion that under steady-state conditions only phenylalkylamine, but not dihydropyridine-sensitive Ca^{2+} channels are functional in PT cells (McCarty et al., 1991b). Since felodipine reduced anoxia-induced increase in $[Ca^{2+}]_i$, additional Ca^{2+}-entry processes must be activated

during anoxia. Most likely, anoxia also results in cell swelling which process activates dihydropyridine-sensitive Ca^{2+} channels, as recently reported by McCarty and O'Neil (1991b).

We show for the first time that anoxia-induced increase in $[Ca^{2+}]_i$ can be partly inhibited by Ca^{2+} channel blockers. D600 (1 μM) and less effectively felodipine (10 μM) reduced anoxic $[Ca^{2+}]_i$ by 20 to 30%. Rather unexpectedly, 100 μM D600 further reduced anoxic $[Ca^{2+}]_i$ up to 60%. It is unlikely that this further reduction is due to a specific effect of D600 on L-type Ca^{2+} channels since at 1 μM inhibition of these channels should be maximal, in view of the I_{50} values for Ca^{2+} channel blockers. It is more likely that 100 μM D600 blocks non-specifically other Ca^{2+} entry pathways, which in the present study could also be blocked by La^{3+} (Fig. 8C). No further efforts were made to identify these other Ca^{2+} pathways, which may either be non-specific leaks or until now unidentified channels. The question whether reduction of anoxia-induced increase in $[Ca^{2+}]_i$ correlates with protection against cell injury cannot be answered from the present study, since the cultured cells were not significantly injured after 60 min of exposure to anoxia. This contrasts with studies on freshly-isolated PTs where the onset of anoxic cell injury occurs much faster (Almeida et al., 1992). However, our observations do not imply that cultured cells are unsuited for studying anoxia related cell injury. We noted that anoxic incubations up to 2 h induced substantial cell injury, suggesting a reduced sensitivity of cultured cells to anoxic injury (to be published). A slower onset of events leading eventually to cell death may even be advantageous in studies aimed to delineate the mechanisms behind these events. Finally, *in vivo*, Ca^{2+} channel blockers do not offer complete protection (Weinberg, 1991; Weinberg et al., 1991a; Almeida et al., 1992), which is in line with the present finding that Ca^{2+} channel blockers only partially reduce anoxia-induced increases in $[Ca^{2+}]_i$.

In conclusion, we demonstrated that the complete absence of O_2 in combination with substrate depletion induces increases in $[Ca^{2+}]_i$ in PT cells in primary culture, which results from Ca^{2+} influx that is partially inhibited by phenylalkylamines and less effectively by dihydropyridines.

CHAPTER 3

Effects of Ca^{2+} channel blockers, low Ca^{2+} medium and glycine on cell Ca^{2+} and injury in anoxic rabbit proximal tubules

U.M. Rose, R.J.M. Bindels, J.W.C.M. Jansen and C.H. Van Os
in: Kidney International (in press)

ABSTRACT

L-type Ca^{2+} channel blockers have been shown to be protective against ischemia-induced injury of the kidney, suggesting that increased $[Ca^{2+}]_i$ plays an important role in the pathogenesis of ischemic cell injury. To assess the role of $[Ca^{2+}]_i$ in anoxic injury of the PT and the protective effect of Ca^{2+} channel blockers, digital imaging fluorescence microscopy was used to monitor $[Ca^{2+}]_i$ in individual PT cells during 60 min of anoxia. $[Ca^{2+}]_i$ started to rise within 10 min and reached maximal levels between 30 to 45 min of anoxia. The onset of this increase and the maximal levels reached varied markedly among individual cells. The mean values for initial and maximal anoxic $[Ca^{2+}]_i$ were 109 ± 2 (N = 209) and 422 ± 14 (N = 240) nM, respectively. Methoxyverapamil (D600; 1 μM) significantly reduced anoxic $[Ca^{2+}]_i$ to 122 ± 5 nM ($P < 0.05$; N = 79). Removal of Ca^{2+}_o, completely abolished anoxia-induced increases in $[Ca^{2+}]_i$, confirming that these increases in $[Ca^{2+}]_i$ result from Ca^{2+} influx. During 60 min of anoxia, PT cells showed a gradual decrease in cell viability to 54 ± 2%. D600 (1 μM) significantly increased cell viability to 64 ± 3% ($P < 0.05$). Glycine (5 mM), however, increased cell viability to 77 ± 4% without a significant reduction in anoxic $[Ca^{2+}]_i$ levels. Low Ca^{2+} medium only protected when 0.1 mM La^{3+} was included, which condition increased cell viability to 82 ± 5%. La^{3+} did not enter PT cells and probably protects via a membrane-stabilizing effect. The combination of glycine and La^{3+} did not further increase protection. In conclusion, D600 almost completely prevented anoxia-induced increases in $[Ca^{2+}]_i$ by blocking Ca^{2+} influx via L-type Ca^{2+} channels. Since D600 only partly protected PT cells against anoxic injury, $[Ca^{2+}]_i$ unrelated cell injury, which is attenuated by glycine, is a more prominent factor in anoxia-induced cell injury in rabbit PT cells.

INTRODUCTION

Disruption of intracellular Ca^{2+} homeostasis may be an important factor in the development of cell injury during ischemia, hypoxia or anoxia (Farber, 1990; Nicotera et al., 1990). $[Ca^{2+}]_i$ has also been suggested to be important in hypoxic injury of renal PTs (Young et al., 1991; Burke et al., 1992). Increases in $[Ca^{2+}]_i$ using ionomycin, induced cell injury in cultured PT cells and in freshly-isolated PTs (Phelps et al., 1989; Weinberg, 1991). Others have argued that $[Ca^{2+}]_i$ is not a prerequisite for cell injury. Jacobs et al. (1991) observed cell injury in PTs during anoxia without significant changes in $[Ca^{2+}]_i$. In contrast, in cultured PT cells, chemical anoxia (Smith et al., 1992) as well as anoxia (Rose et al., 1993) increased $[Ca^{2+}]_i$ without detectable LDH release in the first 60 min of anoxia.

If $[Ca^{2+}]_i$ is a primary mediator of cell injury resulting from O_2 deprivation, than prevention of Ca^{2+} entry into cells should prevent or delay cell death. Indeed, exposure of isolated PTs to 30 min of anoxia in a low Ca^{2+} medium reduced LDH release (Takano et al., 1985; Wetzels et al., 1993), and delayed blebbing (Smith et al., 1992). Moreover L-type Ca^{2+} channel blockers have been shown to reduce ^{45}Ca uptake as well as LDH release after 10 min of hypoxic and anoxic incubation of PTs (Almeida et al., 1992). Although many studies have shown that Ca^{2+} channel blockers can be protective against ischemic renal injury in various circumstances (Burke et al., 1992), there is no general agreement on the question whether Ca^{2+} channel blockers directly protect renal epithelial cells (Bonventre, 1993). So far, very high concentrations of Ca^{2+} channel blockers have been used to show protective effects (Almeida et al., 1992; Wetzels et al., 1993). These high concentrations preclude a specific effect on L-type Ca^{2+} channels and beneficiary effects may be related to nonspecific actions as for example, membrane-stabilization (Katz, 1985). In addition, L-type Ca^{2+} channels have not been demonstrated unequivocally in renal tubular cells (Weinberg, 1991). A recent study on cell volume regulation of PT cells suggests the presence of L-type channels in these cells, since $[Ca^{2+}]_i$ was significantly reduced by 10 μM verapamil (McCarty et al., 1991a). Most importantly, no direct study has been undertaken to investigate the effect of Ca^{2+} channel blockers on $[Ca^{2+}]_i$ during hypoxia or anoxia in PT cells.

In a previous study, we developed a method to investigate increases in $[Ca^{2+}]_i$ in cultured PT cells induced by anoxic periods up to 60 min (Rose et al.,

1993). In that study complete absence of O_2 was realized by inclusion of Oxyrase®, a mixture of oxygenases, to preclude any contribution of reactive oxygen species to cellular injury which may occur in model studies using chemical anoxia or hypoxia (Weinberg, 1991). In cultured PT cells, 1 μM D600 significantly decreased anoxic $[Ca^{2+}]_i$ levels. However, the question whether reduction of anoxia-induced increase in $[Ca^{2+}]_i$ correlated with protection against cell injury could not be answered, since after 60 min of anoxia the cultured PT cells showed no significant increase in LDH release. It is known that in freshly-isolated PTs, cell injury occurs much faster (Almeida et al., 1992). Therefore, we now studied anoxia-induced increases in $[Ca^{2+}]_i$ and the effects of Ca^{2+} channel blockers in freshly-isolated PT cells to find out whether protective effects of Ca^{2+} channel blockers on tubular cells are correlated with a reduction in anoxic $[Ca^{2+}]_i$ levels.

MATERIALS AND METHODS

Isolation of proximal tubules

Rabbit PT cells were isolated by immunodissection as described previously (Rose et al., 1993). Briefly, kidneys were excised from New Zealand white rabbits (≈0.5 kg) A cortical cell suspension, obtained by enzymatic digestion of dissected cortical tissue, was incubated for 60 min on ice with monoclonal antibodies 85C8 and 101E12, recognizing PT cell surface specific antigens. After three washings, the cell suspension was added to goat anti-mouse IgG-coated petri dishes and incubated for 15 min at 20 °C. The dishes were washed carefully and adherent PT cells were scraped off the dishes. The PT cells were collected and resuspended at a density of $1x10^6$ cells/ml in a mixture of Dulbecco's Modified Eagles medium (Imperial #1-466-14, Hampshire, UK): Ham's F12 medium (Gibco, #041-01765M, Paisley, UK) (1:1), supplemented with gentamycin (10 μg/ml), $NaHCO_3$ (25 mM), glutamine (14 mM), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (50 nM), 0.5% (v/v) non-essential amino acids (Gibco, #043-01140H, Paisley, UK), prostaglandin E_1 (70 ng/ml), triiodothyronine (5 pM), Na_2SeO_3 (50 nM) and 5% (v/v) FCS, pH 7.4 (K_1 + 5% FCS medium). PT cell suspension aliquots (5 ml) were incubated at 37 °C in 25 cm^2 tissue culture flasks (Costar 3055, Cambridge, MA, USA) for maximally 6 h in an humidified incubator, gassed with 5% CO_2 in air, and every hour cell viability was estimated. The isolated PT cell preparation consisted mainly of clumps of 3 to 6 cells.

Fura-2 loading of PT cells

Clumps of freshly-isolated PT cells were attached to round coverslips (Ø 22 mm; Menzel, Germany) which were coated with Cell-Tak® (Collaborative Research Incorporated, Bedford, MA, USA) as follows: 5 µl pure Cell-Tak® was brought onto the glass coverslip and dried in air for 1 h at 20 °C. Thereafter 100 µl of a PT cell suspension was added to the dried Cell-Tak® within 30 min at 37 °C. The attached PT cells were loaded with fura-2 AM by incubating the coverslip with PT cells for 1 h at 37 °C in K_1 + 5% FCS medium containing 10 µM fura-2 AM (Molecular Probes, Eugene, OR, USA), 0.02% (w/v) pluronic F127 (Molecular Probes, Eugene, OR, USA), 4% (v/v) FCS and 3 mM probenecid (Sigma, St. Louis, MO, USA). After loading, the PT cells were washed twice in the experimental medium and were used immediately. All experiments were performed in the presence of 0.3 mM probenecid in order to inhibit fura-2 leakage via organic anion transporters.

Measuring $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in single cells was measured by a digital imaging technique making use of a MagiCal system (Applied Imaging Techniques, Tyne & Wear, UK). The fura-2-loaded PT cells were alternatingly excited at 340 and 380 nm and emitted light was captured at 510 nm with a CCD camera followed by digital imaging using TARDIS® software (Applied Imaging Techniques, Tyne & Wear, UK). The 340-380 nm capturing sequence was interrupted by 30 s of no capturing, divided into 10 s excitation at 340 nm allowing for cell focusing, and 20 s of no excitation using a shutter to avoid bleaching. The MagiCal system has been described in detail by Neylon et al. (1990).

$[Ca^{2+}]_i$ was calculated according to the formula derived by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_D \times R_{bf} \times [(R-R_{min}) / (R_{max}-R)]$, where K_D is the dissociation constant of fura-2 for Ca^{2+} of 224 nM; R is the ratio of fluorescence of the cell at 340 and 380 nm; R_{max} and R_{min} represent the ratios of fura-2 fluorescence intensity at 340 and 380 nm excitation obtained by treating the PT cells with 5 µM ionomycin in the presence and absence (estimated by addition of 2 mM EGTA) of Ca^{2+}_o, respectively; R_{bf} is the maximal 380 nm signal divided by the minimal 380 nm signal.

Anoxic chamber experiment

To estimate $[Ca^{2+}]_i$ during anoxia, the same anoxic chamber was used as was described previously (Rose et al., 1993). Briefly, fura-2-loaded PT cells attached to a coverslip were mounted in an anoxic chamber at 37 °C. After filling the anoxic chamber with 100% N_2 gassed modified Krebs-Henseleit buffer (KHB; composition in mM: 138 NaCl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1 l-alanine, 5 l-lactate, 20 HEPES/Tris and 360 mU/ml Oxyrase® (Oxyrase Inc., Ashland, Ohio, USA), pH 7.4). It was mounted on the stage of a Nikon Diaphot inverted microscope and $[Ca^{2+}]_i$ was measured using the MagiCal system. After 45 to 60 min of anoxic incubation, reperfusion was started by introduction of oxygenated KHB con-

taining 10 mM glucose For $[Ca^{2+}]_i$ measurements during anoxia we used at least 4 preparations, and from every preparation at least 2 coverslips were used From one coverslip 8 to 16 cells were monitored and analyzed, which is roughly the number of cells in the viewing field of the CCD camera (3 to 4 clumps of cells)

Estimation of cell viability

Clumps of PT cells attached to Cell-Tak®-coated coverslips were mounted in the anoxic chamber at 37 °C in modified KHB Simultaneously, PO_2 was monitored using a Clark-type oxygen electrode (Rose et al., 1993) After 60 min of anoxic incubation, cell viability was estimated by means of trypan blue or propidium iodide staining To this end, the coverslip with PT cells was incubated for 1-2 min in 0 08% (w/v) trypan blue or 5 µg/ml propidium iodide. The percentage stained cells was determined by means of light microscopy for trypan blue staining or by means of fluorescence microscopy with excitation at 490 nm and 510 nm emission for propidium iodide staining. As controls, several coverslips with PT cells were incubated in the presence of oxygen and substrate. The influence of several conditions on cell viability was tested in such a way that for every condition at least 4 preparations were used. From every preparation at least 2 coverslips were incubated in the anoxic chamber and after 60 min between 100 and 200 cells were counted after staining. The cells were chosen randomly by counting as many cells as possible on one coverslip within 5 min We did not observe a specific pattern of cell killing within clumps of cells.

Materials

Conjugated antibodies were obtained from Sigma (St. Louis, MO, USA). D600 was kindly provided by Knoll AG (Ludwigshafen, Germany). Felodipine was donated by Astra Pharmaceutica BV (Rijswijk, The Netherlands). All chemicals were of the purest grade

Statistical analysis

All reported data are expressed as means ± SE. Statistical analysis was performed on ratio values using analysis of variance ($P < 0.05$ is significant). Subsequently, statistical differences between experimental groups were estimated by means of contrast analysis according to Fisher (Snedecor et al., 1974).

RESULTS

$[Ca^{2+}]_i$ during anoxia

As shown previously, PO_2 in the anoxic chamber decreased rapidly as a result of filling the chamber with hypoxic medium. The residual PO_2 was reduced to zero within 10 min due to the enzyme complex Oxyrase® (Rose et al., 1993). As a

result of substrate-free anoxia, $[Ca^{2+}]_i$ in freshly-isolated PT cells started to rise and reached maximal levels within 45 min (Fig. 1). However, the onset of this rise and the maximal levels reached varied strikingly among individual cells within one preparation (Fig. 1) and subsequent preparations (Fig. 2). This heterogeneity was not only observed in cells from different clumps, but also within one clump. At the introduction of oxygen and glucose, elevated $[Ca^{2+}]_i$ declined rapidly towards initial levels, but in a few cells $[Ca^{2+}]_i$ remained elevated (Fig. 1). In some cells a sudden and abrupt decline in the fluorescence ratio was observed (Fig. 1). Inspection of the 340 and 380 nm fluorescence values indicated a decline in both signals, which means that leakage of fura-2 had occurred due to a loss of cell membrane barrier.

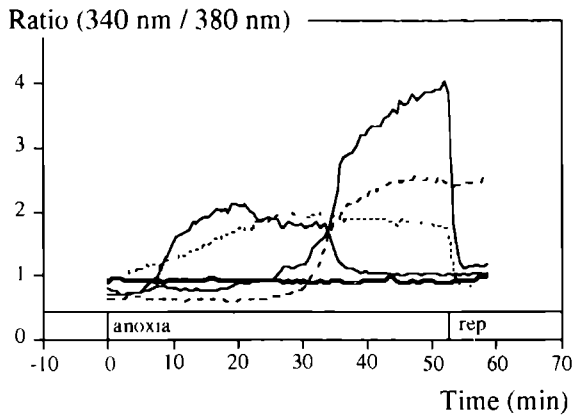


Figure 1

Time-dependency of increase in $[Ca^{2+}]_i$ in four individual PT cells (typical examples) in response to O_2 and glucose deprivation at 37 °C. At -10 min the anoxic chamber is closed and $[Ca^{2+}]_i$ measurements start at 0 min (anoxia). After 52 min of anoxia, O_2 and glucose are reintroduced (reperfusion: rep). In one cell a fall in the fluorescence ratio is observed after 30 min, due to loss of fura-2 fluorescence probe, indicative of cell death. The bold straight line represents a normoxic control experiment. $[Ca^{2+}]_i$ is presented as the 340 and 380 nm ratio of fura-2 excitation.

In figure 2, fura-2 ratios and $[Ca^{2+}]_i$ values are given which have been observed in close to 200 cells: the mean initial level, the individual maximal levels attained, and the mean value 10 min after reintroducing O_2 and substrate. $[Ca^{2+}]_i$ monitoring starts 10 min after filling the anoxic chamber due to mounting the chamber on the stage of the microscope. Since several cells have lost fura-2 fluorescence during the experiment, the maximal stable fura-2 level,

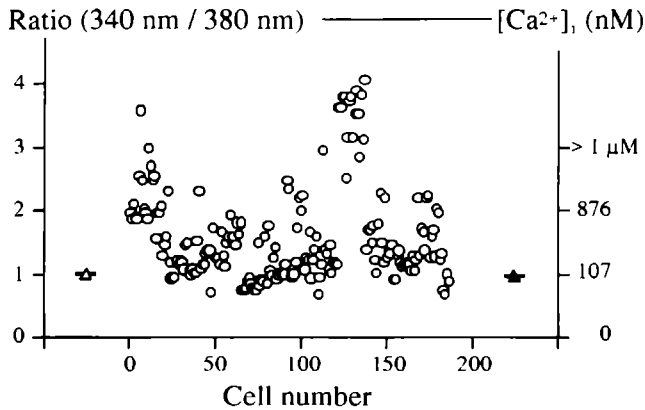


Figure 2

Summary of the heterogeneous responses in $[Ca^{2+}]_i$ of PT cells to anoxic incubation: Δ, mean initial $[Ca^{2+}]_i$ value (± SE) measured immediately after mounting the anoxic chamber on the microscope stage (10 min after filling the anoxic chamber); o, maximal $[Ca^{2+}]_i$ in individual cells reached after 45 min of anoxia; ▲, mean $[Ca^{2+}]_i$ value (± SE) reached 10 min after reintroducing O_2 and glucose. $[Ca^{2+}]_i$ is presented as fura-2 ratio (left) and in nM (right).

reached just before fura-2 started to leak, has been used in calculating the mean anoxic fura-2 ratio. When the fura-2 level had not reached a plateau, then the ratio was excluded. From those cells which had lost fura-2 during the anoxic incubation, the reperfusion fura-2 ratios were also excluded. Therefore, figures 2-7 also contain maximal anoxic fura-2 ratios of cells which did not survive the anoxic incubation. However, the fura-2 ratio is used which was reached just before the dye started to leak. We never observed a sudden increase in fura-2 ratio just before fura-2 started to leak due to cell injury. The mean initial fura-2 ratio measured 10 min after closing the chamber (1.01 ± 0.02, N = 218), is not significantly different from the mean fura-2 ratio 10 min after reperfusion (0.98 ± 0.03, N = 209; P > 0.1).

Phenylalkylamines have been shown to reduce ischemic renal injury when present during an ischemic insult (Burke et al., 1984; Schrier et al., 1987), therefore we tested, whether D600 reduces the rise in $[Ca^{2+}]_i$ during anoxia. Figure 3 shows that the presence of 1 µM D600 during the anoxic period reduced the maximal fura-2 ratio from 1.59 ± 0.06 to 1.04 ± 0.04 (P < 0.05). D600 also slightly, albeit significantly, reduced the initial fura-2 ratio from 1.01 ± 0.02 to 0.89 ± 0.02 (P < 0.05). A similar reduction in the ratio is observed 10 min after reperfusion: from 0.98 ± 0.03 to 0.77 ± 0.03. In addition, the effect of 1 µM felodipine was studied, but this dihydropyridine derivative was unable to

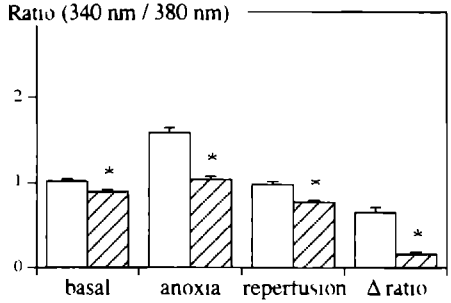


Figure 3
The effect of 1 μM D600 on initial (basal), maximal anoxic, and reperfusion values for fura-2 ratios, and on Δratio values (maximal minus initial ratios). Ratios observed in the presence of D600 (dashed columns) are compared to ratios observed in the absence of D600 (open columns). Columns represent mean ratio values ± SE with N ≥ 32 (*P < 0.05· D600 versus control ratio values).

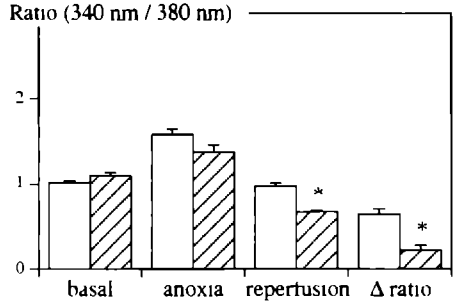


Figure 4
The effect of 1 μM felodipine on initial (basal), maximal anoxic, and reperfusion ratio values, and on Δratio values (maximal minus initial ratios) Experimental ratios (dashed columns) are compared to control ratios (open columns), i.e. anoxic incubation without the addition of felodipine. Columns represent mean ratios ± SE with N = 32 (*P < 0.05: experimental versus control ratio values).

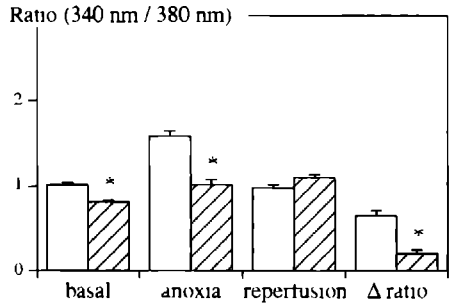


Figure 5
The effect of Ca^{2+}_o exclusion (-Ca^{2+} / +EGTA) on initial (basal), maximal anoxic, and reperfusion ratio values, and on Δratio values (maximal minus initial ratios). Experimental ratios (dashed columns) are compared to control ratios (open columns) i.e. anoxic incubation in the presence of 2 mM $CaCl_2$ without EGTA Columns represent mean ratio values ± SE with N ≥ 38 (*P < 0.05: experimental versus control ratio values).

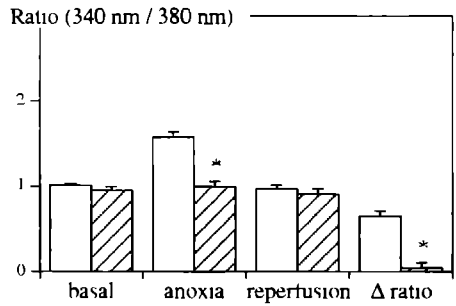


Figure 6
The effect of Ca^{2+}_o exclusion and addition of 0.1 mM $LaCl_3$ (-Ca/+La) on initial (basal), maximal anoxic, and reperfusion ratio values, and on Δratio values (maximal minus initial ratios). Experimental ratios (dashed columns) are compared to control ratios (open columns), i.e. anoxic incubation in the presence of 2 mM $CaCl_2$ without $LaCl_3$. Columns represent mean ratio values ± SE with N ≥ 44 (*P < 0.05: experimental versus control ratio values).

reduce significantly the anoxia-induced increases in the fura-2 ratio (Fig. 4). When, however, the differences in fura-2 ratios between initial and the maximal anoxic levels are considered (Δratio in Fig. 4) it becomes clear that felodipine has a significant effect, albeit not as striking as D600.

Next, the dependence of anoxia-induced increases in $[Ca^{2+}]_i$ on $Ca^{2+}{}_o$ was investigated. We studied two conditions, one in which $Ca^{2+}{}_o$ was reduced below 10^{-8} M by adding 0.5 mM EGTA, and one in which Ca^{2+} was simply omitted from the solution. In the second condition, medium $[Ca^{2+}]$ is still ≈20 μM and therefore 0.1 mM La^{3+} was added to block any residual Ca^{2+} influx. Figures 5 and 6 demonstrate clearly that omitting $Ca^{2+}{}_o$ or preventing Ca^{2+} influx by La^{3+} effectively reduced the anoxia-induced increase in $[Ca^{2+}]_i$, which proves that the rise in $[Ca^{2+}]_i$ stems from Ca^{2+} influx and not from depletion of intracellular Ca^{2+} stores.

Since glycine protects against ischemic injury, the effect of glycine on $[Ca^{2+}]_i$ during anoxia was also studied. The result is shown in figure 7. Whatever the mechanism behind glycine protection may be, it is clear that glycine is unable to influence $[Ca^{2+}]_i$ during an anoxic period of 45 to 60 min.

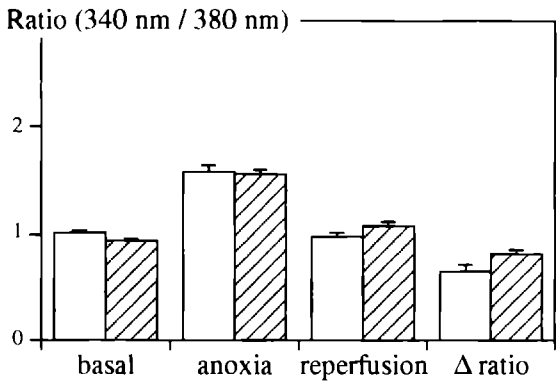


Figure 7
The effect of 5 mM glycine on initial (basal), maximal, and reperfusion ratio values and on Δratio values (maximal minus initial ratios). The experimental ratios (dashed columns) are compared to control ratios (open columns), i.e. anoxic incubation without addition of glycine. Columns represent mean ratio values ± SE with N ≥ 77.

Cell injury

In some cells loss of fura-2, indicative for cell death, was observed during the anoxic incubation. Therefore the effect of anoxia on cell viability was further quantified. Cell injury could not be assessed by measuring LDH release because the number of cells in the anoxic chamber was too small to measure LDH release reproducibly. For this reason, cell viability was estimated with the membrane impermeable nuclear stain propidium iodide and with trypan blue.

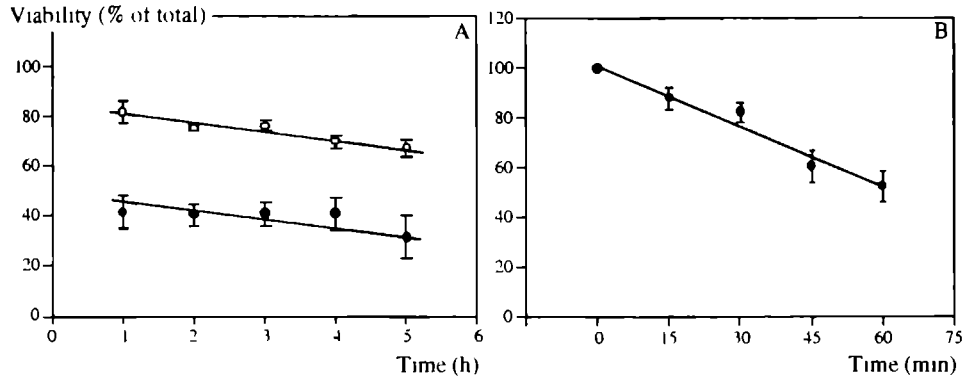


Figure 8

Cell viability of PT cells.

A. Decrease in cell viability (presented as % of total) of PT cells under normoxic and anoxic conditions. o, cell viability of PT cells in normoxic conditions in a humidified incubator as a function of time expired since the cells were isolated (see methods). PT cell isolation takes ± 3 h, which is the zero time point in this graph. •, cell viability of PT cells after 1 h anoxic incubation. To start anoxic incubations, PT cells were taken from the normoxic cell stock at 1 h intervals. For example, data points at 3 h comprise cells which have been in stock for 2 h and incubated for 1 h in the anoxic chamber.

B. Time-dependency of the decrease in cell viability of PT cells during anoxic incubations. Viability is presented as % of total cell count, normalized with respect to the spontaneous loss of viability in the preservation vial (figure 8A).

We observed in 3 preparations that trypan blue and propidium iodide stain the same cells after 60 min of anoxic incubation in the chamber. In further experiments we preferred trypan blue to quantify cell viability for practical reasons. Since cell viability already decreased during preservation of the cells before the anoxic experiments started, a survival curve was made for every isolation (Fig. 8A). After the cell isolation procedure, which takes ± 3 h, cell viability was 84 ± 1%. Cell viability decreased significantly to 67 ± 3% ($P < 0.05$) during 5 h of preservation (Fig. 8A). In figure 8A it is also shown that 1 h of anoxia decreased cell viability with a percentage that is independent of the time expired since the cell isolation started. Therefore, in all experiments presented in figures 9 and 10, the viability of the control group was set at 100%. This normalization procedure allows for direct comparison of protective effects, independent of the time that the experiment was started. In this way presented, cell viability decreased after 60 min of anoxia to 54 ± 2%. In figure 8B it is shown that cell death gradually increases with the time of exposure to anoxia.

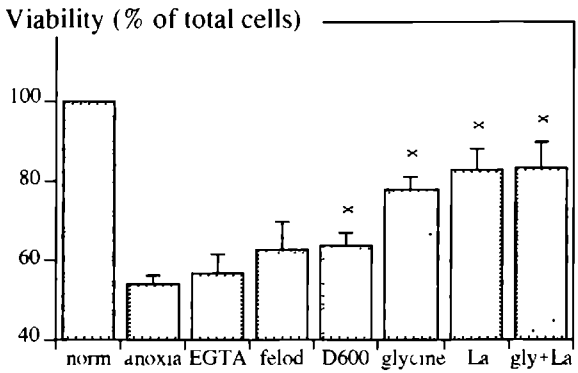


Figure 9

The effects of D600 (1 μM), felodipine (felod, 1 μM), Ca^{2+}-free medium plus 0.5 mM EGTA (EGTA), Ca^{2+}-free medium plus 0.1 mM $LaCl_3$ (La), glycine (5 mM) and the combination glycine, Ca^{2+}-free plus 0.1 mM $LaCl_3$ (gly+La) on the viability of PT cells after 60 min of anoxia. Experimental values are compared to control values (i.e. anoxic incubation in KHB medium without glucose). Viability, estimated by trypan blue staining, is presented as % of total cell count. Values represent mean values ± SE with $N \geq 8$ (*$P < 0.05$, experimentalversus control values).

In figure 9 the effects of D600, felodipine, extracellular Ca^{2+}-free solutions and glycine on cell viability after 60 min of anoxic incubation are tabulated. D600 significantly increased cell viability to 64 ± 3% ($P < 0.05$), but the protective effect is small compared to the impressive effect on $[Ca^{2+}]_i$ during anoxia (Fig. 3). The protective effect of Ca^{2+}-free solutions appear not to be unambigious. When Ca^{2+}_o is complexed with 0.5 mM EGTA there is no significant protection against anoxic injury ($P > 0.1$). On the other hand when 0.1 mM La^{3+} is added to prevent influx of residual Ca^{2+}_o during anoxia, cell viability increased from 54 ± 2% to 82 ± 5% ($P < 0.05$). In 3 separate experiments, we tested the protective effect of low Ca^{2-} alone, but found no significant improvement in cell viability (data not shown). The effects of low Ca^{2+} and of low Ca^{2+} plus 0.1 mM La^{3+} were also tested on the normoxic control group, but both conditions did not significantly influence cell viability ($P > 0.1$). Glycine during anoxia significantly increased cell viability up to 77 ± 4% ($P < 0.05$). This protection occurs despite the elevated anoxic $[Ca^{2+}]_i$ levels. The combination of glycine and 0.1 mM La^{3+} further increased cell viability to 83 ± 7% ($P < 0.05$), albeit this further increase is not significant with respect to glycine or La^{3+} alone ($P > 0.1$).

Figure 10 summarizes the observed effects of D600, Ca^{2+}-free solution and glycine on anoxia-induced increases in $[Ca^{2+}]_i$ and on cell viability after 60 min of anoxia. This figure demonstrates a clear dissociation between protective effects and effects on $[Ca^{2+}]_i$, suggesting a minor role for $[Ca^{2+}]_i$ in the pathogenesis of anoxic injury of freshly-isolated PT cells.

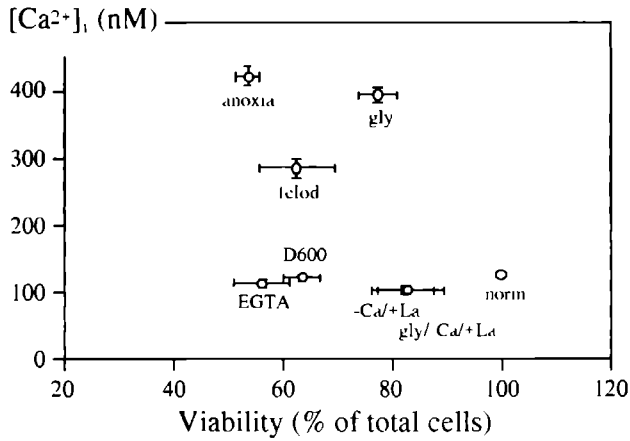


Figure 10
Correlation between $[Ca^{2+}]_i$ levels reached during 60 min of anoxia and the protective effects, expressed as cell viability, of D600, felodipine, Ca^{2+}-free solutions, and glycine. Values represent mean ± SE ($N \geq 8$; $^*P < 0.05$).

DISCUSSION

In the present study, freshly-isolated PT cells were subjected to 60 min of anoxia. $[Ca^{2+}]_i$ measurements showed anoxia-induced increases in $[Ca^{2+}]_i$ which were heterogeneously in onset and in the maximal level reached. The Ca^{2+} channel blocker, D600 (1 μM) reduced almost completely the anoxia-induced increase in $[Ca^{2+}]_i$, but exhibited a moderate protective effect.

Cytosolic Ca^{2+} in PT cells could be measured conveniently after attachment of the cells to a coverslip with Cell-Tak®. This substance did not induce cell injury, but made it possible to follow single PT cells during an anoxic period of 60 min. Several individual PT cells within one preparation were monitored using digital imaging fluorescence microscopy. Some investigators have previously used cell suspensions (Jacobs et al., 1991;

Almeida et al., 1992) in which responses of several thousands of tubules are summed. The present study demonstrated that anoxia-induced increases in $[Ca^{2+}]_i$ are rather heterogeneous at the single cell level. Nevertheless, maximal $[Ca^{2+}]_i$ levels were always reached before 45 min of anoxia, with a mean value of 422 ± 14 (N = 240). In view of a Ca^{2+}_o concentration of 2 mM, this anoxic $[Ca^{2+}]_i$ value represents a relatively modest increase. Anoxic incubation precludes mitochondrial Ca^{2+} loading due to the complete absence of oxidative phosphorylation and a driving force for Ca^{2+} uptake. Nonetheless, our results point to stabilization of the anoxic $[Ca^{2+}]_i$ value below the extracellular level, suggesting the presence of some kind of endogenous autoprotective mechanism, whereby the ATP-depleted state decreases the Ca^{2+} permeability of the plasma membrane preventing the cell from being flooded with Ca^{2+}. The anoxic $[Ca^{2+}]_i$ value reported previously for cultured PT cells (Rose et al., 1993) was even higher than the one observed in freshly-isolated cells (662 ± 22 nM versus 422 ± 14 nM). Other studies using cultured PT cells also reported modest increases in $[Ca^{2+}]_i$ after inhibition of metabolism (McCoy et al., 1988; Phelps et al., 1989).

The complete suppression of anoxia-induced increases in $[Ca^{2+}]_i$ by removing Ca^{2+}_o suggests that intracellular Ca^{2+} stores do not contribute to Ca^{2+} overload during anoxia. This phenomenon has also been observed in cultured PT cells (Smith et al., 1992 ; Rose et al., 1993). A possible explanation could be that when intracellular stores begin to release Ca^{2+}, the plasma membrane Ca^{2+}-ATPase may still operate at low ATP concentrations and an early increase in $[Ca^{2+}]_i$ activates calmodulin and thereby stimulates the plasma membrane Ca^{2+}-ATPase.

The present study provides functional evidence for the presence of L-type Ca^{2+} channels in freshly-isolated PT cells. The phenylalkylamine D600 almost completely abolished anoxia-induced increases in $[Ca^{2+}]_i$, at a concentration of 1 μM. Previously, in cultured PT cells a reduction of elevated $[Ca^{2+}]_i$ by 1 μM D600 has been shown (Rose et al., 1993), but the effect was less profound, suggesting that L-type Ca^{2+} channels are either less important in anoxia-induced increases in $[Ca^{2+}]_i$ or are less expressed in cultured PT cells. Felodipine, a dihydropyridine, was less effective than D600 which was also previously reported for cultured PT cells (Rose et al., 1993). This finding confirms the observation of McCarty et al. (1991a, 1991b), that verapamil but not nifedipine reduced resting $[Ca^{2+}]_i$ in isolated PTs. Verapamil has been described to inhibit

Na^+-Ca^{2+} exchange activity in cardiac sarcolemmal membrane vesicles with an IC_{50} of approximately 200 µM (Kaczorowski et al., 1989). Because in PT cells a complete reduction of anoxic $[Ca^{2+}]_i$ is realized at 1 µM D600, inhibition of Ca^{2+} influx via Na^+-Ca^{2+} exchange is an unrealistic alternative. In addition, Na^+-Ca^{2+} exchange is most likely absent from PT cells which was shown elegantly with molecular biological techniques (Yu et al., 1992) and immunohistochemical techniques (Reilly et al., 1993).

Almeida et al. (1992) reported recently that anoxia-induced increase in ^{45}Ca uptake and LDH release in rat PTs was inhibited by verapamil during 10 min of anoxia. During longer anoxic incubations the time course of ^{45}Ca uptake reached a plateau phase. We observed that D600 prevents increases in $[Ca^{2+}]_i$ during 60 min of anoxia. Hence, the fura-2 imaging technique provides information over a much longer experimental period than conventional isotope uptake experiments.

Besides $[Ca^{2+}]_i$ measurements, cell viability after 60 min anoxia was determined. In contrast to cultured PT cells (Rose et al., 1993), freshly-isolated PT cells exhibit significant cell injury within 15 min of anoxia, which was demonstrated by trypan blue or propidium iodide staining and loss of fura-2 fluorescence. This clearly demonstrates that freshly-isolated PT cells are more sensitive to anoxic injury than cultured PT cells. Differences in hypoxia tolerance have also been described between hepatoma cells and hepatocytes (Hugo-Wisseman et al., 1991).

Exposure of PT cells to 60 min of anoxia in the absence of Ca^{2+}_o did not improve cell viability. Takano et al. (1985) reported, that reduction of $[Ca^{2+}]_o$ to around 2.5 µM attenuated cell injury in short-term anoxia of rabbit PTs. Either the longer anoxic incubation or the addition of EGTA may have resulted in loss of protection in our study. There is, however, no agreement whether lowering of $[Ca^{2+}]_o$ offers protection (Bonventre, 1993). For example, hepatocytes as well as cardiomyocytes were more susceptible to anoxic injury when Ca^{2+}_o was less than 10 µM (Smith et al., 1981; Cheung et al., 1982). Surprisingly, addition of 0.1 mM La^{3+} to a nominally Ca^{2+}-free solution protected PT cells as effectively as glycine against anoxic injury. From the fura-2 ratio in figure 6 it is concluded, that La^{3+} does not enter the cells during anoxic incubation, since fura-2 exhibits a similar response to La^{3+} as to Ca^{2+}. Therefore, this novel protecting effect of small amounts of La^{3+} is exerted extracellularly, most likely via stabilization of

the plasma membrane resulting in a lower susceptibility to lysis by enzymes which are activated during anoxia.

Using PTs and cultured PT cells, Weinberg and colleagues found that glycine protects against cell injury associated with chemical anoxia, ouabain and $[Ca^{2+}]_i$ (Weinberg, 1991). Also in our study glycine provided protection in the absence of reducing anoxia-induced increases in $[Ca^{2+}]_i$. Since the mechanism of this protection is still unknown, it is of interest that the protective effects of glycine and La^{3+} were not additive. This could mean that La^{3+} and glycine prevent a common factor from exerting its injurious effect. Hypothetically, when La^{3+} stabilizes the plasma membrane preventing enzymatic lysis then glycine could possibly inhibit those very enzymes which cause membrane lysis. Searching for such a common factor could be of help in uncovering the mechanism of glycine protection against cell injury.

In conclusion, anoxia-induced increases in $[Ca^{2+}]_i$ in freshly-isolated PT cells result from Ca^{2+} influx via L-type Ca^{2+} channels. Despite the fact that anoxia-induced increases in $[Ca^{2+}]_i$ are completely suppressed by 1 μM D600, this Ca^{2+} channel blocker offers moderate protection suggesting a minor role of $[Ca^{2+}]_i$ in the pathogenesis of anoxic cell injury.

CHAPTER 4

Cellular acidification occurs during anoxia in cultured but not in freshly-isolated rabbit proximal tubular cells

U.M. Rose, S.L. Abrahamse, J.W.C.M. Jansen, R.J.M. Bindels and C.H. Van Os
in: Pflügers Archiv (submitted)

ABSTRACT

In a variety of cells it has been shown that acidosis is protective against anoxic injury. We demonstrated previously that PT cells in primary culture were more resistant to anoxia-induced cell injury than freshly-isolated cells. Therefore, we asked the question whether a difference in cellular acidification during anoxia could explain this difference in susceptibility to anoxia. To answer this question, pH_i was measured during anoxic incubation of PT cells in culture and freshly-isolated. PT cells were incubated in an anoxic chamber at 37 °C after loading with BCECF-AM or fura-2 AM. pH_i and $[Ca^{2+}]_i$ were measured by digital imaging fluorescence microscopy. During anoxia, pH_i in cultured PT cells decreased from 7.3 ± 0.1 to 6.8 ± 0.1, whereas pH_i in freshly-isolated cells did not significantly decrease. During 1 h of anoxia, cell viability of freshly-isolated PT cells decreased significantly to 54 ± 2% ($P < 0.05$) while no loss in viability was observed in cultured PT cells. Clamping the pH_i during anoxia at 6.7 and 6.1 significantly increased cell viability in freshly-isolated PT cells to 76 ± 5% and 72 ± 4%, respectively ($P < 0.05$). In addition, the intrinsic buffering capacity ($ß_i$) in cultured and freshly-isolated PT cells were determined and turned out to be not different at $pH_i \geq 7.3$. Below pH_i 7.3, $ß_i$ increased several-fold in freshly-isolated PT cells, and rose to significantly higher levels in cultured PT cells. During anoxia, $[Ca^{2+}]_i$ increased from 118 ± 2 to 662 ± 22 nM in cultured cells and from 111 ± 16 to 512 ± 33 nM in freshly-isolated cells. A pH_i clamp at 6.1 significantly reduced anoxic $[Ca^{2+}]_i$ to 412 ± 5 nM in freshly-isolated PT cells ($P < 0.05$).

In conclusion, the development of cytosolic acidosis during anoxia protects cultured PT cells against anoxia-induced cell injury. The fact that freshly-isolated PT cells do not acidify during anoxia must be due to less glycolytic activity and to a greater $ß_i$.

INTRODUCTION

ATP depletion and acidosis are prominent features during hypoxic or ischemic insults in many tissues, including the kidney (Weinberg et al., 1991a). In general, ATP depletion results in the disturbance of intracellular ion homeostasis which leads eventually to cell damage (Kehrer et al., 1990; Weinberg, 1991). Increased $[Ca^{2+}]_i$ has also been suggested to mediate injury during ATP depletion in several organ systems, including kidney (Burnier et al., 1988; Shanley et al., 1991; Weinberg, 1991; Weinberg et al., 1991a), liver (Bonventre et al., 1985; Sakaida et al., 1991), brain (Bickler, 1992) and heart (Hayashi et al., 1992). It has been shown that acidification of hypoxic tissue, resulting from glycolytic lactate production, ATP hydrolysis and CO_2 accumulation, can enhance resistance to damaging effects of oxygen deprivation in kidney (Burnier et al., 1988; Shanley et al., 1988; Weinberg 1985, 1991; Weinberg et al., 1991a; Zager et al., 1993), cardiomyocytes (Bond et al., 1991; Koop et al., 1992), and hepatocytes (Gores et al., 1988, 1989b; Harrison et al., 1991; Nieminen et al., 1990). However, the mechanism behind protection offered by lowering the pH is unknown. In some cells such as cardiomyocytes, acidosis has an energy-conserving effect caused by a smaller energy demand (Koop et al., 1992). In addition, pH_i plays a role in the preservation of ion gradients across the plasma membrane during ATP depletion by decreasing plasma membrane conductance pathways of, for example, Ca^{2+} or inhibit Ca^{2+}-calmodulin-regulated processes (Weinberg, 1985). Moreover, mechanisms responsible for membrane and cell damage such as phospholipid and protein degradation by phospholipases and proteases, appear to be pH-dependent with maximal activity at or near physiological pH and minimal activity at acidotic pH (Harrison et al., 1991). Protection by extracellular acidosis is mediated by intracellular acidification in hepatocytes (Bronk et al., 1991), but until now this has not been confirmed in renal cells (Burnier et al., 1988; Shanley et al., 1988; Weinberg, 1985, 1991; Weinberg et al., 1991a; Zager et al., 1993).

Previous studies on anoxia-induced cell injury in PT cells revealed a striking difference in sensitivity to anoxia between freshly-isolated and cultured PT cells (Rose et al., 1993, 1994). The fact that cultured cells were more resistant to anoxia could be due to the presence of a protective factor, as for example intracellular acidosis. The objective of the present study was to

measure pH_i and $[Ca^{2+}]_i$ during anoxia in cultured and freshly-isolated PT cells and to investigate whether cytosolic acidosis plays a role in protection against anoxia-induced cell injury in PT cells.

MATERIALS AND METHODS

Isolation of proximal tubule cells

Rabbit kidney PT cells were isolated by immunodissection as described previously (Rose et al., 1993). Briefly, kidneys were excised from New Zealand white rabbits (≈0.5 kg). A cortical cell suspension, obtained by enzymatic digestion of dissected cortical tissue, was incubated for 60 min on ice with monoclonal antibodies 85C8 and 101E12, recognizing cell surface antigens specific for the PT. After three washings, the cell suspension was added to goat anti-mouse IgG-coated petri dishes and incubated for 15 min at 20 °C. The dishes were washed carefully and adherent PT cells were collected and resuspended in a mixture of Dulbecco's Modified Eagles medium (Imperial #1-466-14, Hampshire, UK): Ham's F12 medium (Gibco, #041-01765M, Paisley, UK) (1:1), supplemented with gentamycin (10 µg/ml), $NaHCO_3$ (25 mM), glutamine (14 mM), insulin (5 µg/ml), transferrin (5 µg/ml), hydrocortisone (50 nM), 0.5% (v/v) non-essential amino acids (Gibco, #043-01140H, Paisley, UK), prostaglandin E_1 (70 ng/ml), triiodothyronine (5 pM), Na_2SeO_3 (50 nM), pH 7.4; hereafter this medium is referred to as K_1 medium. PT cells were either used directly or cultured. To obtain a primary culture, PT cells were seeded on collagen-coated coverslips (Ø 22 mm; Menzel, Germany) at a density of $2x10^5$ cells/cm^2. Cells were grown to confluency in K_1 medium, supplemented with 5% (v/v) FCS during the first 24 h of culture. For experiments with cultured PT cells, the PT cells were used 5 to 6 days after seeding. In separate experiments, freshly-isolated PT cells were resuspended at a density of $1x10^6$ cells/ml K_1 + 5% (v/v) FCS, and 5 ml aliquots were incubated at 37 °C in 25 cm^2 tissue culture flasks (Costar 3055, Cambridge, MA, USA). Both cultured and freshly-isolated PT cells were kept at 37 °C in a humidified incubator under 5% CO_2 in air until use.

Fura-2 and BCECF loading of PT cells

Freshly-isolated PT cells were attached to Cell-Tak® (Collaborative Research Incorporated, Bedford, MA, USA) coated round coverslips (Ø 22 mm; Menzel, Germany) as follows: 5 µl pure Cell-Tak® on a glass coverslip was dried in air for 1 h at 20 °C, and 100 µl of a PT cell suspension was applied to the Cell-Tak® coated coverslip within 30 min at 37 °C. Cultured PT cells or attached freshly-isolated PT cells were loaded with fura-2 by incubating the coverslip with PT cells for 1 h at 37 °C in K_1 + 5% FCS medium containing 5 and 10 µM fura-2 AM (Molecular Probes, Eugene, OR, USA) for cultured and freshly-isolated PT cells respectively, 0.02% (w/v) pluronic F127 (Molecular Probes, Eugene, OR, USA), 4% (v/v)

FCS and 3 mM probenecid (Sigma, St. Louis, MO, USA). PT cells were loaded with BCECF-AM (Molecular Probes, Eugene, OR, USA) by incubating the coverslips for 45 min at 37 °C in K_I + 5% FCS medium containing 2 µM BCECF-AM and 3 mM probenecid (Sigma, St. Louis, MO, USA). Thereafter PT cells were incubated 30 min in K_I + 5% FCS at 37 °C for de esterification. After loading and de-esterification, the PT cells were washed twice in the experimental medium and were used immediately. All experiments were performed in the presence of 0.3 mM probenecid to inhibit fura-2 or BCECF leakage.

$[Ca^{2+}]_i$ and pH_i measurements

Fura-2 fluorescence was monitored using digital imaging equipment (MagiCal, Applied Imaging Systems, Tyne & Wear, UK). The fura-2-loaded PT cells were alternatingly excited at 340 and 380 nm and emitted light was captured at 510 nm with a CCD camera followed by digital imaging using TARDIS® software (Applied Imaging International Ltd., Tyne & Wear, UK). The 340-380 nm capturing sequence was interrupted by 30 s of no capturing, divided into 10 s excitation at 340 nm allowing for cell focusing, and 20 s of no excitation using a shutter to avoid bleaching The MagiCal system has been described in detail by Neylon et al.(1990).

$[Ca^{2+}]_i$ was calculated according to the formula derived by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_D \times R_{bf} \times [(R-R_{min}) / (R_{max}-R)]$, where K_D is the dissociation constant of fura-2 for Ca^{2+} of 224 nM; R is the ratio of fluorescence of the cell at 340 and 380 nm; R_{max} and R_{min} represent the ratios of fura 2 fluorescence intensity at 340 and 380 nm excitation obtained by treating the PT cells with 5 µM ionomycin in the presence and absence (obtained by addition of 2 mM EGTA) of Ca^{2+}_o, respectively; R_{bf} is the maximal 380 nm signal divided by the minimal 380 nm signal

BCECF fluorescence was measured by using a Newcastle Photonic system (NPS; Newcastle, UK) in which a photomultiplier is connected to a Nikon Diaphot inverted microscope. The BCECF-loaded PT cells were alternatingly excited at 490 and 440 nm and emitted light was collected at 1 s intervals at 520 nm. BCECF ratios were converted to pH_i by clamping cell pH in a high-$[K^+]$ medium, containing (in mM): 112 potassium gluconate, 28 KCl, 10 NaCl, 1 $MgCl_2$, 0.01 $CaCl_2$, 5 HEPES, 10 D-glucose, 20 mannitol and 0 01 nigericin. The medium pH was varied between 6.0 and 7 9 by addition of HCl or Tris. Individual calibrations were pooled and a mean calibration curve was calculated by means of linear regression.

Intrinsic buffering capacity of PT cells

The intrinsic buffering capacity ($ß_i$) was estimated using a method described by Boyarski et al. (1988). Briefly, all mechanisms for pH_i regulation in the PT cells were inhibited by omitting Na^+ and HCO_3 from the perfusate (composition in mM· 140 NMGCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5 l-alanine, 10 D-glucose, pH 7.4). After 6 min, the PT cells were exposed for 4 min to the same solution except that now 40 mM NMGCl was substituted

by 40 mM NH_4Cl. Subsequently, the NH_4Cl concentration was stepwise lowered to 20, 10, 5 and 0 mM at 4 min intervals. Each experiment was followed by a pH_i calibration according to the procedure described above. $ß_i$ was calculated from the pH_i change induced by reducing the NH_4Cl concentration.

Anoxic chamber experiment

Anoxic conditions were realized in an anoxic chamber as described previously (Rose et al., 1993). Fura-2- or BCECF-loaded PT cells on coverslips were mounted in this anoxic chamber at 37 °C. After filling the anoxic chamber with 100% N_2 gassed modified Krebs-Henseleit Buffer (KHB; composition in mM: 138 NaCl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1 l-alanine, 5 l-lactate, 20 HEPES/Tris and 360 mU/ml Oxyrase® (Oxyrase Inc., Ashland, Ohio, USA)), $[Ca^{2+}]_i$ and pH_i were monitored for 45-60 min.

Estimation of cell viability

PT cells were mounted in the anoxic chamber under continuous monitoring of PO_2 using a Clark-type electrode (Rose et al., 1993). After anoxic incubations, cell viability was estimated by means of LDH leakage for cultured cells (Rose et al., 1993) and by means of trypan blue or propidium iodide staining for freshly-isolated cells (Rose et al , 1994). For LDH activity measurements, medium and cells were collected separately. To this end, cells were scraped off the coverslip in 1 ml MilliQ® (Millipore) water and the resulting cell suspension was sonicated for 30 s at 100 Watt to release all LDH Both cell and medium samples were centrifuged for 5 min at 200 x g and LDH content was measured as described previously (Rose et al., 1993). For trypan blue and/or propidium iodide staining, coverslips with PT cells were incubated for 1-2 min in 0.08% (w/v) trypan blue or 5 µg/ml propidium iodide. The percentage stained cells was determined by counting using light microscopy for trypan blue staining or by using fluorescence microscopy for propidium iodide staining as described previously (Rose et al., 1994).

Materials

All chemicals were of the purest grade and obtained from Sigma (St. Louis. MO, USA) unless otherwise indicated.

Statistical analysis

All reported data are expressed as means ± SE. Statistical analysis was performed on ratio and viability values using analysis of variance ($P < 0.05$ is significant). Subsequently, statistical differences between experimental groups were estimated by means of contrast analysis according to Fisher (Snedecor et al., 1974).

RESULTS

$[Ca^{2+}]_i$ and pH_i during anoxia

$[Ca^{2+}]_i$ and pH_i were monitored during substrate-free anoxia in cultured and freshly-isolated PT cells. Figure 1A shows a typical example of cultured PT cells, where anoxia induced increases in $[Ca^{2+}]_i$ within 10 min, reaching a maximal level within 30 min. After reintroducing oxygen and glucose, i.e. reperfusion, $[Ca^{2+}]_i$ returned completely to basal levels, as reported previously (Rose et al., 1993). In cultured PT cells, anoxia also induced a decrease in pH_i. The ratio of 490 nm and 440 nm declined significantly from 3.11 ± 0.10 to 2.44 ± 0.11 ($P < 0.05$) within the first 15 min of anoxia. This level was maintained throughout the entire anoxic period until reperfusion. At reperfusion the BCECF ratio value increased significantly to 3.04 ± 0.11 ($P < 0.05$). Figure 1B shows a typical experiment in which anoxia induced an increase in $[Ca^{2+}]_i$ in freshly-isolated PT cells. Upon reperfusion $[Ca^{2+}]_i$ not always returned to initial levels, as described before (Table I and Rose et al., 1994). In contrast to cultured PT cells, pH_i did not decrease significantly during 45 min of anoxia in freshly-isolated PT cells. In order to provide real pH_i values, a calibration was performed in both cultured and freshly-isolated PT cells. PT cells were incubated in a high-$[K^+]$ medium with a pH ranging from pH 6.0 to 7.9. Cultured PT cells were calibrated directly after the anoxic experiment. In some freshly-isolated PT cells this was not feasible since anoxia had induced cell

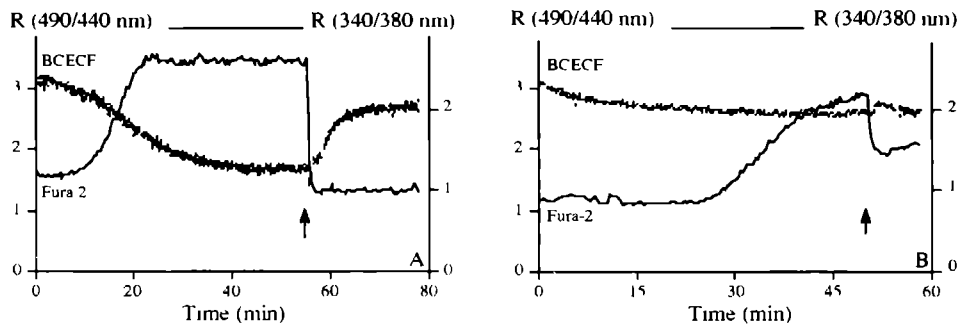


Figure 1

Typical $[Ca^{2+}]_i$ and pH_i responses of cultured (**A**) and freshly-isolated (**B**) PT cells during substrate-free anoxic incubation. Anoxia induced $[Ca^{2+}]_i$ increases in both cultured and freshly-isolated PT cells. However, pH_i only decreased in cultured PT cells. At reperfusion (arrow) the low pH_i increased to the basal level in cultured PT cells. $[Ca^{2+}]_i$ is presented as 340 and 380 nm fura-2 ratios and pH_i as 490 and 440 BCECF ratios.

injury and all BCECF fluorescence was lost into the medium. In these cases pH calibrations were performed in separate experiments. Figure 2 shows pH calibration curves for cultured (Fig. 2A) and freshly-isolated (Fig. 2B) PT cells. Both curves were linear in the pH range used. From these calibration curves, pH_i values were calculated for cultured and freshly-isolated PT cells.

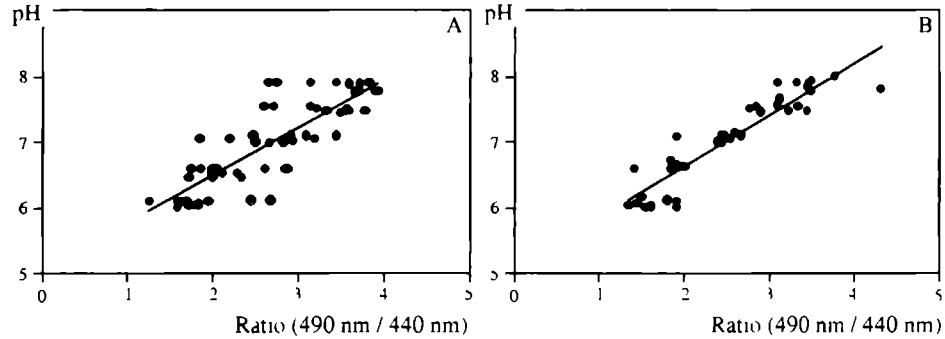


Figure 2
Calibration curves of pH for cultured (**A**) and freshly-isolated (**B**) PT cells. PT cells were incubated in high-$[K^+]$ buffer containing 10 μM nigericin with pH ranging from 6.0 to 7.9. The calibration curves for cultured and freshly-isolated PT cells are pH = 4.831 + (0.763 x R) and pH = 5.096 + (0.775 x R), respectively, with pH is the buffer pH and R is the 490 and 440 nm ratio value. The correlation coefficients are 0.93 and 0.92 for cultured and freshly-isolated PT cells, respectively.

Figure 3 gives the pH_i changes which occurred during anoxia and subsequent reperfusion. In cultured PT cells, pH_i decreased significantly from pH 7.3 ± 0.1 to pH 6.8 ± 0.1 during anoxia ($P < 0.05$) and increases again significantly to pH 7.2 ± 0.1 ($P < 0.05$) upon reperfusion, while in freshly-isolated PT cells, pH_i did not change significantly ($P > 0.1$).

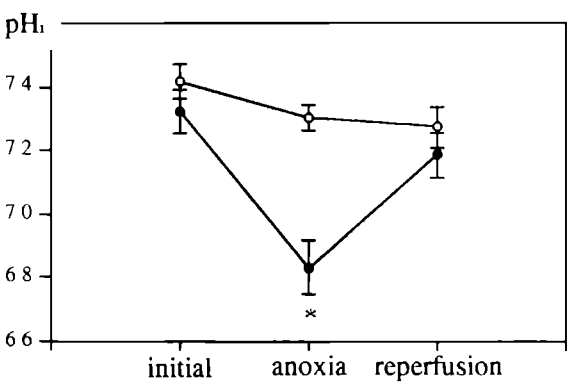


Figure 3
pH_i of cultured (•) and freshly-isolated (o) PT cells measured at initial, which is 10 min after filling the anoxic chamber, anoxic, which is during the steady-state reached after 20 min of anoxia, and reperfusion, which is at the introduction of oxygen and substrate, conditions. Each point represents the mean pH_i ± SE with $N \geq 8$ (*$P < 0.05$: anoxic versus basal pH_i).

Protection by cytosolic acidosis

Cell viability of cultured PT cells after 60 min of anoxic incubation was unaltered, as reported previously (Rose et al., 1993). Cell viability of freshly-isolated PT cells, however, significantly decreased to 54 ± 2% ($P < 0.05$). To test whether intracellular acidosis protects against anoxic injury, pH_i was clamped at low pH_o during 60 min of anoxia. To this end, freshly-isolated PT cells were incubated in substrate-free high-[K^+] medium of pH 6.6 or 6.0 containing 10 μM nigericin. Figure 4 shows a typical experiment of clamping the pH_i at 6.0. During anoxic incubation, the BCECF ratio was stabilized at 1.23 which corresponds with a pH_i of 6.0. On average, pH_i was 6.7 ± 0.1 and 6.1 ± 0.1 during clamping in pH_o 6.6 and 6.0, respectively. After 60 min of anoxic incubation at pH_o 6.6 or 6.0, cell viability was significantly higher than in the control situation (Fig. 5). In addition, we tried to clamp pH_i of cultured PT cells during anoxia at 7.3 to see whether this protocol induced cell injury. However, pH_i measurements revealed that in cultured PT cells we were unable to clamp pH_i since the cells still acidified during anoxia. Even in the presence of valinomycin or amiloride pH_i could not successfully be clamped at pH_i 7.3.

In Table I the influence of cytosolic acidosis on $[Ca^{2+}]_i$ is summarized for freshly-isolated PT cells. It is shown that intracellular acidosis increased initial $[Ca^{2+}]_i$ significantly, but on the other hand maximal anoxic $[Ca^{2+}]_i$ was significantly reduced when compared to anoxia in the absence of acidosis. The mean $[Ca^{2+}]_i$ after 10 min of reperfusion is significantly higher than the initial level, independent of the pH_i during anoxia.

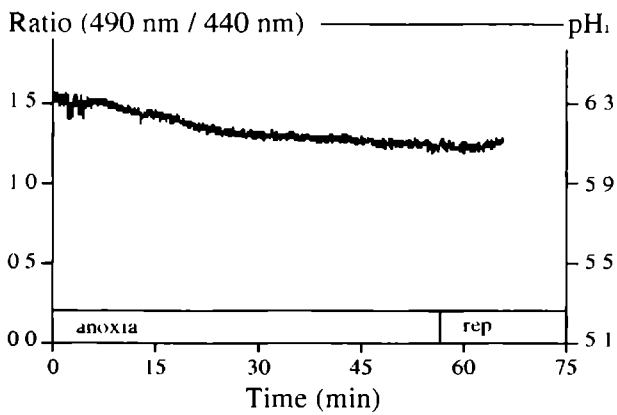


Figure 4
Typical pH_i trace of pH_i clamp of freshly-isolated PT cells during anoxic incubation in substrate-free high-[K^+] buffer of pH 6.0, containing 10 μM nigericin. During this pH clamp, the 490/440 nm ratio declines to 1.23. pH_i is presented as 490/440 nm ratios as well as real pH values.

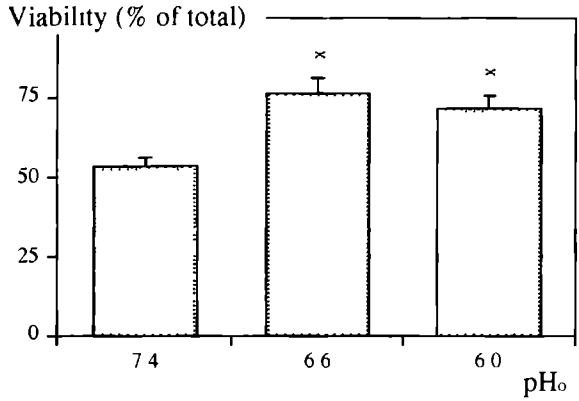


Figure 5
Cell viability of freshly-isolated PT cells after 60 min of anoxic incubation in substrate free KHB of pH 7.4 (pH_o=7.4) or in substrate-free pH clamp buffer (pH_o=6.6 and pH_o=6.0). Columns represent mean viability values ± SE with N ≥ 19 (*P < 0.05: pH_o is 6 6 and 6.0 versus pH_o is 7.4).

Table I. The effect of intracellular acidosis on $[Ca^{2+}]_i$ in freshly-isolated PT cells

Condition	$[Ca^{2+}]_i$ (nM)	
	pH_o=7 4	pH_o=6.0
Initial	111 ± 16	150 ± 1#
Anoxia	512 ± 33*	412 ± 5*#
Reperfusion	184 ± 14*	213 ± 7*

Anoxic incubation of freshly-isolated PT cells at normal and low pH medium Initial $[Ca^{2+}]_i$, which is $[Ca^{2+}]_i$ 10 min after filling the chamber, anoxic $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ 10 min after reperfusion, are presented in nM. All data are mean values ± SE of at least 5 experiments. (*P < 0.05· anoxia and reperfusion values versus pre-anoxic values; # P < 0 05: pH_o=6.0 versus pH_o=7.4).

Estimation of intrinsic buffering capacity $ß_i$

A factor which could explain the difference in pH_i values in cultured and freshly-isolated PT cells during anoxic incubations is the $ß_i$ for both cell types. Therefore, $ß_i$ was estimated. Figure 6 shows $ß_i$ values as a function of pH_i. In the pH range 8.0 to 7.3, $ß_i$ values were similar for cultured and freshly-isolated PT cells. However, at lower pH_i, $ß_i$ values in freshly-isolated PT cells started to increase and to deviate significantly from those measured in cultured cells. Nevertheless, the absence of cytosolic acidosis during anoxia in freshly-isolated PT cells cannot be explained solely on the basis of a higher $ß_i$ at lower pH_i.

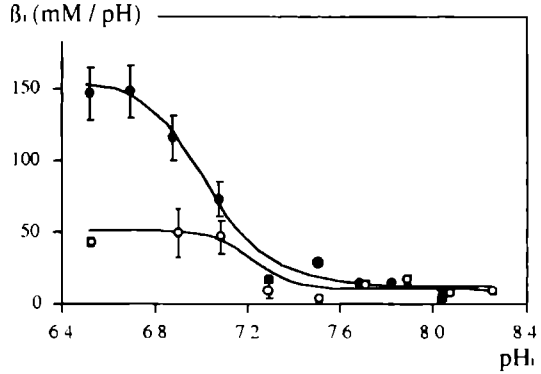


Figure 6
Intrinsic buffering capacity ($ß_i$) of cultured (o) and freshly-isolated (•) PT cells. At $pH_i < 7.0$, $ß_i$ of freshly-isolated PT cells is significantly higher than of cultured PT cells ($P < 0.05$). Results represent mean pH_i and $ß_i \pm SE$ with $N > 6$.

DISCUSSION

The present study demonstrates that during anoxic incubation intracellular acidosis develops in cultured PT cells but does not occur in freshly-isolated PT cells. In addition, intracellular acidosis was shown to protect against anoxia-induced cell injury. Therefore, we conclude that the absence of cytosolic acidosis explains, at least partly, why freshly-isolated PT cells are more prone to anoxia-induced cell injury.

In vivo ischemic studies reported that acidosis, caused by ATP hydrolysis and accumulation of CO_2, protected tissues against cell injury (Shanley et al., 1988). Until now, *in vitro* experiments have only demonstrated a protective effect of extracellular acidosis against hypoxia/anoxia-induced cell injury in kidney (Burnier et al., 1988; Shanley et al., 1988 Weinberg, 1985, 1991; Weinberg et al., 1991a; Zager et al., 1993), hepatocytes (Kehrer et al., 1990) and cardiomyocytes (Bond et al., 1991). In these studies however, it was not shown that protection by low pH_o is mediated via intracellular acidosis. Our study demonstrates, that anoxia caused intracellular acidosis in cultured PT cells but not in freshly-isolated PT cells. Cultured cells, in general, are more glycolytic than freshly-isolated cells and therefore substrates in the cytosol at the onset of anoxia may be metabolized, forming lactic acid and ATP. In addition, hydrolysis of ATP leads to an intracellular acid load and thus to cytosolic acidification (Busa et al., 1984). In contrast, non-cultured PT cells have less glycolytic capacity and apparently do not form lactic acid during anoxia (Dickman et al., 1990). Moreover, we showed that freshly-isolated PT cells have a higher $ß_i$ at

pH_i around 7.3 than cultured PT cells and may therefore buffer more strongly pH_i during anoxic incubation. By subjecting freshly-isolated PT cells to low pH clamp buffer during anoxia, it could be shown that acidosis is also protective in these cells, obviously mediated by intracellular acidosis since pH_i clamping experiments resulted in pH_i values of 6.7 and 6.1. The fact that anoxia induced cell death in freshly-isolated PT cells but not in cultured PT cells is most likely due to the occurrence of acidosis in cultured cells only.

There are several mechanisms by which acidosis may protect against anoxia-induced cell death.

Firstly, hypoxia/anoxia-induced phospholipid degradation has been reported to be pH-dependent (Harrison et al., 1991). Also ATP depletion, in chemical anoxia, resulted in a loss of phospholipid mass and increased permeability of the plasma membrane and of cellular organelles (Weinberg et al., 1991a).

Secondly, low pH was shown to preserve metabolic energy in cardiomyocytes (Hayashi et al., 1992; Koop et al., 1992). In PTs however, no increase in cellular ATP content was found in anoxia at low pH_o compared to pH_o 7.4 (Weinberg, 1985; Kehrer et al., 1990; Nieminen et al., 1990; Zager et al., 1993). In our experiments ATP preservation is also unlikely, since anoxia induced increases in $[Ca^{2+}]_i$ in cultured PT cells, in spite of cytosolic acidosis. Increases in $[Ca^{2+}]_i$ occur only after ATP depletion.

Thirdly, acidosis may preserve ion gradients across the plasma membrane during anoxia. Cytosolic acidosis decreases plasma membrane conductance pathways and, as a result, cellular K^+ does not decrease during chemical anoxia in hepatocytes (Bronk et al., 1991) or during anoxia in hepatocytes and rabbit PTs (Pentilla et al., 1974). Another gradient which is influenced by pH is the Ca^{2+} gradient (Weinberg, 1985). Under normal physiological conditions, pH_i and $[Ca^{2+}]_i$ appear to be closely linked: a decrease in pH_i reduces transmembrane Ca^{2+} fluxes in cardiomyocytes and an increase in pH_i promotes Ca^{2+}-mediated processes during differentiation of the cell (Weinberg, 1985). Low pH_i has also been shown to inhibit Ca^{2+} influx across the plasma membrane in kidney (Shanley et al., 1991) and cardiomyocytes (Hayashi et al., 1992). Burnier et al. (1988) described elevated ^{45}Ca uptake associated with cell injury during hypoxia in PTs. This hypoxia-induced cell injury could be prevented by acidosis which also abolished elevated ^{45}Ca uptake. Others have

reported that Ca^{2+}-mediated damage caused by hypoxia can be inhibited by acidosis in a dose-dependent fashion, i.e. protection afforded by acidosis could be overcome by increasing perfusate Ca^{2+} and the injury caused by elevated Ca^{2+} could be reduced by a further decrease in pH (Shanley et al., 1991). In the present study it was shown that acidosis slightly increased initial $[Ca^{2+}]_i$ levels in freshly-isolated PT cells. This effect may be due to competition between H^+ and Ca^{2+} for intracellular Ca^{2+} binding sites. During anoxia, however, $[Ca^{2+}]_i$ levels were slightly reduced by exposure to acidosis. This result is in line with the notion that acidosis reduces Ca^{2+} influx across the plasma membrane (Burnier et al., 1988; Shanley et al., 1991), because we demonstrated previously that anoxia-induced increases in $[Ca^{2+}]_i$ resulted from Ca^{2+} influx (Rose et al., 1993, 1994). However, it is rather unlikely that the protective effect of acidosis is due to the small decrease in $[Ca^{2+}]_i$ levels. In freshly-isolated PT cells it has been shown previously that anoxic cell injury and $[Ca^{2+}]_i$ levels are not directly related (Rose et al., 1993, 1994). Ca^{2+} channel blockers like D600 were shown to reduce anoxic $[Ca^{2+}]_i$ to normoxic levels with only small protective effects. In contrast, glycine protected against anoxic injury to the same extent as acidosis in the present study, but without an effect on anoxic $[Ca^{2-}]_i$ levels (Rose et al., 1994).

Finally, it has been shown that acidosis stabilizes cell membranes (Bell et al., 1971). We have previously shown that 0.1 mM extracellular La^{3+} protects freshly-isolated PT cells against anoxic injury via membrane stabilization (Rose et al., 1994). The effect of acidosis in the present study and the effect of 0.1 mM La^{3+} in the previous study on cell viability are of similar magnitude.

In conclusion, freshly-isolated PT cells are more susceptible to anoxic injury than cultured PT cells, because cytosolic acidosis does not develop during anoxic incubations. The absence of cytosolic acidosis can be explained by a combination of a larger $ß_i$ and less glycolytic capacity of freshly-isolated PT cells in comparison to cultured PT cells.

CHAPTER 5

The role of calcium in cell injury induced by chemical anoxia in proximal tubular cells in primary culture

U.M. Rose, J.W.C.M. Jansen, R.J.M. Bindels and C.H. Van Os
in: Cell Calcium (submitted)

ABSTRACT

Primary cultures of PT cells were incubated with the mitochondrial uncoupler CCCP (15 μM) in glucose-free medium (chemical anoxia). LDH release after 2 h of incubation with CCCP in glucose-free medium was 15 ± 2%, whereas LDH release after incubation in glucose-free medium alone was 11 ± 2% ($P > 0.1$). During 2 h of chemical anoxia no change in $[Ca^{2+}]_i$ could be detected until the Ca^{2+} ionophore ionomycin (5 μM) was added in addition to CCCP. In the presence of both ionophores, $[Ca^{2+}]_i$ increased significantly from 97 ± 6 nM to well above 1 μM ($P < 0.05$) and LDH release increased to 79 ± 4% after 2 h ($P < 0.05$). Ionomycin alone induced significant increases in $[Ca^{2+}]_i$ exceeding 1 μM, and also increased LDH release to 58 ± 5% after 2 h ($P < 0.05$). However, exclusion of $Ca^{2+}{}_o$ from the incubation medium, only partly decreased LDH release to 44 ± 4% and 39 ± 4% for incubations in the presence of ionomycin or ionomycin + CCCP, respectively ($P < 0.05$). Therefore, chemical anoxia-induced cell injury is only in part Ca^{2+} dependent. Addition of 5 mM glycine to the experimental medium did not prevent increases in $[Ca^{2+}]_i$ induced by ionomycin + CCCP, but significantly reduced LDH release from 79 ± 4% to 20 ± 4% after 2 h ($P < 0.05$). In conclusion, chemical anoxia in combination with ionomycin induced substantially more cell injury in cultured PT cells than the absence of O_2. Moreover, ionomycin induces cell injury which is only partly Ca^{2+}-dependent but mainly results from an undefined toxic effect of this ionophore on cultured PT cells.

INTRODUCTION

Ischemia involves cessation of perfusion of tissues, leading to substrate and oxygen deprivation (Weinberg, 1991). As a result, ATP depletion occurs which results in the disturbance of intracellular ion homeostasis and eventually in loss of cell viability. *In vitro*, several models are being used to mimic ATP depletion in *in vivo* ischemia. During hypoxia, which indicates a state in which oxygenation is reduced below the critical partial oxygen pressure (PO_2) (Weinberg, 1991), mitochondrial respiration is inhibited or even abolished, leading to ATP depletion and induction of cell damage. In hypoxic studies the presence of traces of O_2 results in the formation of reactive oxygen metabolites (ROM) which are likely to induce additional injury (Dawson et al., 1993). Since anoxic conditions, in which total elimination of O_2 from the media is required, are difficult to maintain, in many studies so-called chemical anoxia is used in which metabolic inhibitors induce severe ATP depletion (McCoy et al., 1988; Bellomo et al., 1991; Cannon et al., 1991; Weinberg et al., 1991b, 1992; Marsh et al., 1993b). As in hypoxic studies, the presence of oxygen also leads to ROM production (Dawson et al., 1993).

Previously we used an *in vitro* anoxic model to study cell injury in primary cultures of PT cells (Rose et al., 1993) and freshly-isolated PT cells (Rose et al., 1994). We demonstrated that substrate-free anoxia only caused cell death in freshly-isolated PT cells after 60 min of anoxia. In contrast, $[Ca^{2+}]_i$ increased to comparable levels in cultured and freshly-isolated cells during anoxic incubation. In addition, we reported that anoxia-induced cell death in freshly-isolated PT cells was only in part Ca^{2+}-dependent. Also, others have shown substantial differences between cultured and freshly-isolated cells with respect to tolerance to ATP depletion (Hugo-Wisseman et al., 1991). Phelps et al. (1989) reported that chemical anoxia induced cell death in cultured PT cells within 60 min, while 1 h of anoxia did not result in measurable cell injury in our study (Rose et al., 1993). Furthermore, chemical anoxia has been used as a model for studying cell injury in hepatocytes (Gores et al., 1988, 1989b; Nieminen et al., 1990; Cannon et al., 1991; Harrison et al., 1991; Marsh et al., 1993b), kidney epithelial cells (Snowdowne et al., 1985; McCoy et al., 1988; Weinberg et al., 1991a, 1991b; Smith et al., 1992) and in endothelial cells (Weinberg et al., 1992).

In the present study, we used chemical anoxia to investigate the Ca^{2+}-dependency of cell injury in PT cells in primary culture. In some chemical anoxia studies Ca^{2+} ionophores have been used in addition to metabolic inhibitors to induce high $[Ca^{2+}]_i$. In our previous studies we observed that anoxia-induced increases in $[Ca^{2+}]_i$ were relatively modest. Therefore, we now used Ca^{2+} ionophores to study the deleterious effects of high $[Ca^{2+}]_i$ in addition to ATP depletion.

MATERIALS AND METHODS

Primary cultures of PT cells

Rabbit kidney PT cells were isolated by immunodissection and subsequently cultured as described previously (Rose et al., 1993). Briefly, kidneys were excised from New Zealand white rabbits (≈0.5 kg). A cortical cell suspension, obtained by enzymatic digestion of dissected cortical tissue, was incubated for 60 min on ice with monoclonal antibodies 85C8 and 101E12, recognizing cell surface antigens specific for the PT. After three washings, the cell suspension was added to goat anti-mouse IgG-coated petri dishes and incubated for 15 min at 20 °C. The dishes were washed carefully and adherent PT cells were scraped off the dishes. The immunodissected PT cells were seeded on collagen-coated round glass coverslips (Ø 22 mm; Menzel, Germany) or on collagen-coated 24-well plates (Costar, Badhoevedorp, The Netherlands) at a density of $2x10^5$ cells/cm^2. Cells were grown to confluency in a mixture of Dulbecco's Modified Eagles medium (DMEM, Imperial #1-466-14, Hampshire, UK): Ham's F12 medium (Gibco, #041-01765M, Paisley, UK) (1:1), supplemented with gentamycin (10 µg/ml), $NaHCO_3$ (25 mM), glutamine (14 mM), insulin (5 µg/ml), transferrin (5 µg/ml), hydrocortisone (50 nM), 0.5% (v/v) non-essential amino acids (Gibco, #043-01140H, Paisley, UK), prostaglandin E_1 (70 ng/ml), triiodothyronine (5 pM), Na_2SeO_3 (50 nM), pH 7.4; hereafter this culture medium is referred to as K_1 medium. During the first 24 h of culture, 5% (v/v) FCS was present in the K_1 medium. For all experiments, PT cells were used 4 to 6 days after seeding.

Experimental Design

To start experiments, the plates with the PT cell monolayers were washed three times with phosphate-buffered saline (PBS; composition in mM: 137 NaCl, 8.4 $Na_2HPO_4{\cdot}2H_2O$, 1.5 KH_2PO_4, 2.7 KCl, pH 7.4), and 0.5 ml of modified Krebs-Henseleit buffer (KHB: composition in mM: 138 NaCl, 5 KCl, 1 $MgSO_4$, 1.25 $CaCl_2$, 5 l-lactate, 20 HEPES/Tris), pH 7.4) was placed in the 24-well plates. Next, the test agents glycine or EGTA were added. At Ca^{2+}-free conditions, no $CaCl_2$ was present in the modified KHB. The plates were returned in the humidified incubator at 37 °C gassed with 5% CO_2 in air. After 60 min of preincubation, the ionophores were added: 15 µM CCCP, 5 µM ionomycin, or the combination CCCP +

ionomycin. Plates were then returned to the incubator for 30 to 120 min of experimental incubation. Finally, medium and cells were collected separately for LDH activity measurements. To this end, PT cells were scraped off the plates in 0.5 ml MilliQ® (Millipore) water.

Estimation of LDH activity

LDH activity was measured in the cells and medium. First, the cell fraction was sonicated for 30 s at 100 W to release all LDH from the cells. Cell and medium fractions were both centrifuged at 200 x g for 5 min, and the LDH activity was measured by incubating a sample of the supernatant in 0.6 mM pyruvate and 0.18 mM reduced NADH. The decrease in NADH, a marker for LDH activity, was monitored at 340 nm in a DW 2000 spectrophotometer (SLM Instruments, Urbana, IU, USA). All results are presented as % LDH of total LDH, which is the sum of LDH activity in cells and medium.

Fura-2 loading of PT cell monolayers

PT cells were loaded with fura-2 by incubating coverslips with PT cell monolayers for 1 h at 37 °C in K_1 medium containing 5 μM fura-2 AM (Molecular Probes, Eugene, OR, USA), 0.02% (w/v) pluronic F127 (Molecular Probes, Eugene, OR, USA), 4% (v/v) FCS and 3 mM probenecid (Sigma, St. Louis, MO, USA). After loading, the PT cells were washed in the experimental medium and were used immediately. All experiments were performed in the presence of 0.3 mM probenecid in order to inhibit fura-2 leakage via anion exchangers.

Measuring $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in PT cells was measured by quantitative fluorescence microscopy using the Newcastle Photonic system (NPS; Newcastle, UK). This system contains a photomultiplier tube which is connected to a Nikon Diaphot inverted microscope with a 40x quartz oil immersion objective to monitor single cells. The photomultiplier contains a pin-hole diaphragm to regulate the viewing field. The data in this study represent fluorescence measurements obtained from 2 to 4 cells, captured at 400x magnification. The fura-2 loaded PT cells were alternatingly excited at 340 and 380 nm, and emitted light was collected at 1-s intervals at 510 nm. $[Ca^{2+}]_i$ was calculated according to the formula derived by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_D \times R_{bf} \times [(R-R_{min}) / (R_{max}-R)]$, where K_D is the dissociation constant of fura-2 for Ca^{2+} of 224 nM; R is the ratio of fluorescence of the cell at 340 and 380 nm; R_{max} and R_{min} represent the ratios of fura-2 fluorescence intensity at 340 and 380 nm excitation obtained by treating the PT cells with 5 μM ionomycin in the presence and absence (estimated by addition of 2 mM EGTA) of Ca^{2+}_o, respectively; R_{bf} is the maximal 380 nm signal divided by the minimal 380 nm signal.

Materials

All chemicals were of the purest grade and obtained from Sigma (St. Louis, MO, USA), unless otherwise stated.

Statistical analysis

All reported data are expressed as means ± SE. Statistical analysis was performed on ratio and LDH values using analysis of variance ($P < 0.05$ is significant). Subsequently, statistical differences between experimental groups were estimated by means of contrast analysis according to Fisher (Snedecor et al., 1974).

RESULTS

Ionophore-induced LDH release

The effects of CCCP, ionomycin and CCCP + ionomycin are presented in figure 1A. After 120 min of incubation in glucose-free medium, PT cell monolayers exhibited a LDH release of 11 ± 2%. Incubation with CCCP in glucose-free medium induced no additional LDH release (15 ± 2%; $P > 0.1$). However, incubation with ionomycin alone, or CCCP + ionomycin showed a significant increase in LDH release already after 30 min ($P < 0.05$). The largest LDH release was obtained after 120 min with CCCP + ionomycin. After 120 min, LDH release was 11 ± 2%, 15 ± 2%, 58 ± 4%, and 79 ± 4% for control, CCCP, ionomycin and CCCP + ionomycin incubation, respectively. Addition of 5 mM glycine abolished LDH release resulting from CCCP or ionomycin almost completely (Fig. 1B). CCCP + ionomycin-induced LDH release was significantly reduced from 79 ± 4% to 20 ± 4% after 120 min ($P < 0.05$).

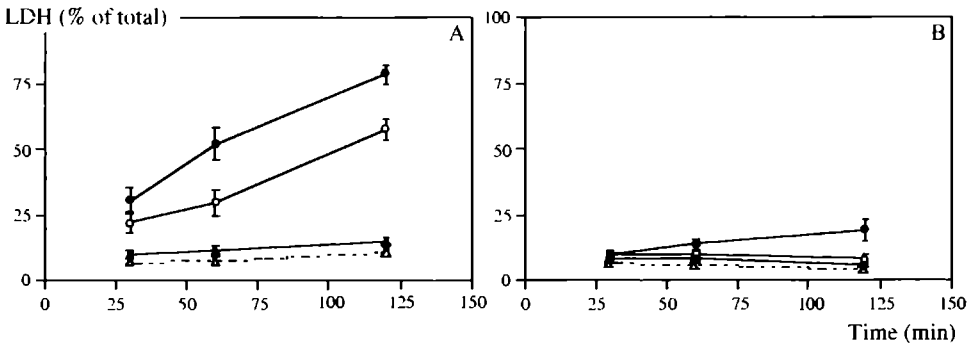


Figure 1

The effects of glucose-free medium (Δ) CCCP (▲; 15 μM), ionomycin (o; 5 μM) and CCCP + ionomycin (●) in glucose-free medium on LDH release from PT cells in the absence (**A**) and presence (**B**) of 5 mM glycine. PT cells were incubated in glucose-free KHB containing the various ionophores for 30, 60 or 120 min at 37 °C. For the experiments in the presence of glycine, the PT cells were preincubated 30 min with 5 mM glycine. LDH release is expressed as % of total cell LDH. Results represent mean values ± SE with $N > 9$.

Next, the dependence of chemical anoxia and ionomycin-induced increases in LDH release on $Ca^{2+}{}_o$ was investigated. PT cell monolayers were incubated in a medium containing the ionophores CCCP and ionomycin for 120 min in normal $[Ca^{2+}]_o$ (1.25 mM), or in Ca^{2+}-free plus 2 mM EGTA ($[Ca^{2+}]_o \leq 10^{-8}$ M) medium. Figure 2 shows that reducing $[Ca^{2+}]_o$ from 1.25 mM to 10^{-8} M significantly increased LDH release from 11 ± 2% to 21 ± 2% ($P < 0.05$) in control PT cells (Fig. 2A), indicating that total exclusion of Ca^{2+} induces cell injury. In contrast, low $[Ca^{2+}]_o$ had protective effects against ionomycin (Fig. 2C) and CCCP + ionomycin (Fig. 2D) induced LDH release. The absence of $Ca^{2+}{}_o$ did, however, not completely prevent LDH release as glycine did. Therefore, cell injury induced by ionomycin is only partly due to high $[Ca^{2+}]_i$ and mainly to an undefined toxic effect.

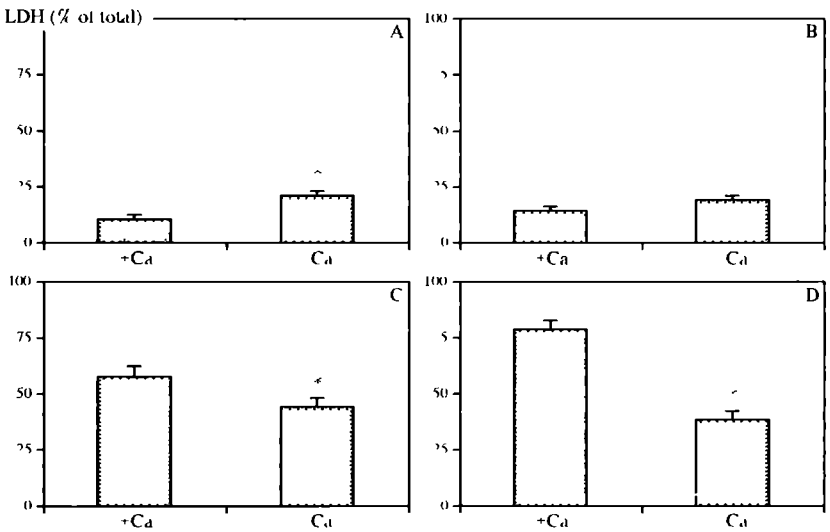


Figure 2

The effects of normal Ca^{2+} containing medium (+Ca) and Ca^{2+}-free medium in addition with 2 mM EGTA (-Ca). LDH release from the PT cell monolayer is estimated after 120 min of incubation in glucose-free control medium (**A**), glucose-free medium with CCCP (15 μM, **B**), with ionomycin (10 μM; **C**), or with CCCP (15 μM) and ionomycin (10 μM; **D**) LDH release is expressed as % of total cell LDH. Results represent mean values ± SE with $N > 9$ (*$P < 0.05$: -Ca versus +Ca).

$[Ca^{2+}]_i$ in chemical anoxia

$[Ca^{2+}]_i$ was measured in PT cell monolayers during incubation with CCCP, ionomycin and CCCP + ionomycin. We previously reported that $[Ca^{2+}]_i$ in primary cultures of PT cells is 118 ± 2 nM (Rose et al., 1993). Figure 3 shows 340 and 380 nm fluorescence ratios as a measure for $[Ca^{2+}]_i$ during chemical anoxia in PT cell monolayers. Addition of 15 μM CCCP to glucose-free medium during 2 h had no significant effect on $[Ca^{2+}]_i$. Ionomycin (5 μM), however, significantly increased the fura-2 ratio from 1.22 ± 0.07 to 3.29 ± 0.20 ($P < 0.05$). A similar fura-2 ratio was found for the combination CCCP + ionomycin, where the ratio increased to 4.23 ± 0.73 ($P < 0.05$). After calibration, $[Ca^{2+}]_i$ was 126 ± 16 nM, > 1 μM and > 1 μM in CCCP, ionomycin and CCCP + ionomycin, respectively. Next, the effect of glycine on $[Ca^{2+}]_i$ was studied during chemical anoxia. Since CCCP + ionomycin induced the largest LDH release, the effect of glycine on $[Ca^{2+}]_i$ was only estimated for the combination CCCP + ionomycin. Figure 3 demonstrates that 5 mM glycine had no effect on elevated $[Ca^{2+}]_i$, indicating that glycine protects against chemical anoxia-induced cell injury, in spite of high $[Ca^{2+}]_i$ levels.

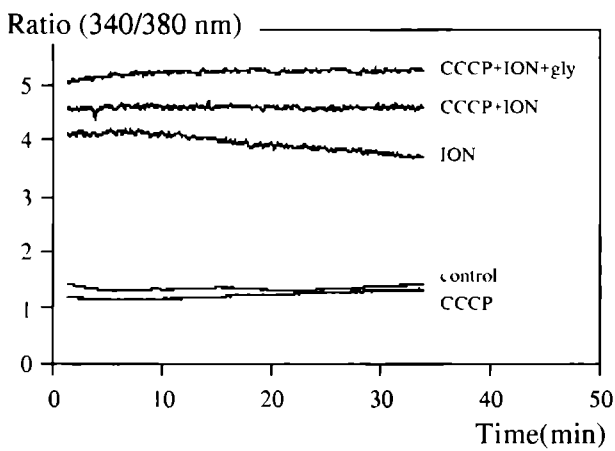


Figure 3

The effects of glucose-free medium (control), CCCP (15 μM; CCCP), ionomycin (5 μM; ION), CCCP + ionomycin (15 and 5 μM; CCCP+ION) and CCCP + ionomycin (15 and 5 μM) in combination with 5 mM glycine (CCCP+ION+gly) on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ is presented as the 340 and 380 nm ratio of fura-2 fluorescence. Each trace represents the fura-2 ratio of 2 to 4 cells from a typical experiment with PT cell monolayers.

DISCUSSION

The present study demonstrated that chemical anoxia in combination with the Ca^{2+} ionophore ionomycin induced cell injury in cultured PT cells which is only partly Ca^{2+}-dependent. The protective effect of glycine, however, was observed in the presence and absence of high $[Ca^{2+}]_i$.

As reported previously, PT cells in primary culture are more glycolytic than non-cultured PT cells, and the presence of glucose during anoxia was sufficient to prevent ATP-depletion and increases in $[Ca^{2+}]_i$ (Rose et al., 1993). Therefore, also in the present study glucose was omitted from the incubation medium. Nevertheless, no change in $[Ca^{2+}]_i$ was observed during 2 h of chemical anoxia elicited by CCCP. Others have demonstrated that incubation in CCCP containing, but glucose-free medium resulted in ATP depletion in MDCK and LLC-PK_1 cells (Weinberg et al., 1991b). This ATP-depleted state was, however, not severe enough to cause an increase in $[Ca^{2+}]_i$ or a loss of cell viability. Similar results were obtained with endothelial cell cultures (Weinberg et al., 1992). In contrast to chemical anoxia, substrate-free anoxia, i.e. the complete absence of O_2, induced an increase in $[Ca^{2+}]_i$ in PT cells in primary culture (Rose et al., 1993). These results indicate that ATP depletion in anoxia is more severe than in chemical anoxia. However, others reported that metabolic inhibitors resulted in cell injury which was accompanied by elevated $[Ca^{2+}]_i$ in cultured PT cells (Phelps et al., 1989; Smith et al., 1992) or to cell death in freshly-isolated PTs (Weinberg et al., 1991a). Chemical anoxia also induced cell death in cultured (Gores et al., 1988, 1989b; Nieminen et al., 1988; Harrison et al., 1991) and freshly-isolated (Nieminen et al., 1990; Cannon et al., 1991; Dickson et al., 1992; Marsh et al., 1993b) hepatocytes.

In the present study, it was shown that the Ca^{2+} ionophore ionomycin in addition to CCCP induces large increases in $[Ca^{2+}]_i$ which are accompanied by additional cell injury. Weinberg et al. (1991b) reported previously similar effects of CCCP + ionomycin in renal epithelial cell lines and in endothelial cell cultures (Weinberg et al., 1992). In our study, incubation with ionomycin alone induced already LDH release, albeit less than the combination CCCP + ionomycin. In MDCK and LLC-PK_1 cells no differences between incubation in ionomycin or in CCCP + ionomycin were observed, which led to the conclusion that ATP could not ameliorate injury induced by high $[Ca^{2+}]_i$. Ionomycin or

A23187-induced cell death has been reported in cultured PT cells (Smith et al., 1992), in freshly-isolated PT cells (Weinberg et al., 1991a), in cultured (Farber, 1990) and in freshly-isolated hepatocytes (Nieminen et al., 1990; Bellomo et al., 1991; Marsh et al., 1993b). Moreover, it was demonstrated that the severe structural and metabolic damage produced by the sustained elevated $[Ca^{2+}]_i$ induced by ionomycin overwhelmed any possible benefit that could be expected from glycolytic ATP production (Weinberg et al., 1991b).

Since incubation in CCCP + ionomycin induced LDH release, it was most likely to assume that the cell injury is Ca^{2+}-dependent. Quite surprisingly, however, CCCP + ionomycin-induced LDH release could only partly be decreased by complexing Ca^{2+}_o with EGTA. This result indicates that in addition to Ca^{2+}-dependent factors also Ca^{2+}-independent factors are involved in initiating cell injury by ionomycin. Omission of Ca^{2+}_o, however, has also cell injuring properties. We observed that total exclusion of Ca^{2+}_o by addition of EGTA slightly increased LDH release in the absence of CCCP or ionomycin. Also others reported cell damaging effects of a low Ca^{2+} medium in freshly-isolated hepatocytes (Thomas et al., 1988a, 1988b), PT cells (Mandel et al., 1984; Takano et al., 1985) and cardiomyocytes (Cheung et al., 1982). In contrast, low Ca^{2+}_o medium potentiated chemical anoxia-induced cell death in neuronal tissue (Verity et al., 1991) and cultured hepatocytes (Smith et al., 1981).

Since chemical anoxia is often used to mimic deleterious effects of ischemia, it is imperative to compare the effects of chemical anoxia with effects of anoxia or ischemia. We previously described an *in vitro* anoxia model for investigating anoxia-induced cell damage in primary PT cell cultures (Rose et al., 1993). As with incubation in CCCP, no change in cell viability was found for up to 1 h of anoxia. However, anoxia caused an increase in $[Ca^{2+}]_i$ from 118 ± 2 to 662 ± 22 nM whereas no change in $[Ca^{2+}]_i$ was found during chemical anoxia with CCCP. When chemical anoxia (CCCP) was combined with ionomycin than $[Ca^{2+}]_i$ shoots up well above 1 μM and this combination induced cell injury. In this situation, CCCP + ionomycin $[Ca^{2+}]_i$ was much higher than during anoxia. Most importantly, the Ca^{2+} ionophore ionomycin exhibited injurious properties on its own, in the absence of high $[Ca^{2+}]_i$.

In the present study we showed that glycine completely abolished ionomycin-induced LDH release and greatly reduced LDH release caused by the combination ionomycin + CCCP. This confirms previous results obtained with

MDCK and LLC-PK_1 cell lines (Weinberg et al., 1991b) and endothelial cells (Weinberg et al., 1992). In freshly-isolated hepatocytes, however, glycine only protected against ionomycin-induced cell injury during the first 15 min of incubation, since the protection was lost after 30 and 60 min of incubation (Marsh et al., 1993b). The deleterious effects of the metabolic inhibitors KCN or CCCP were suppressed by glycine for a much longer period (Marsh et al., 1993b). In the present study, glycine provided protection without reducing elevated $[Ca^{2+}]_i$, which has also been reported by Weinberg et al. (1991b) in MDCK and LLC-PK_1 cells and in freshly-isolated PT cells during anoxia (Rose et al., 1994).

In conclusion, 2 h of chemical anoxia, elicited by CCCP alone, has no effects on cell viability of cultured PT cells. When CCCP and ionomycin were combined, cell injury increased dramatically. However, the increase in cell injury induced by ionomycin was only partly Ca^{2+}-dependent and mainly due to an unspecified toxic effect.

CHAPTER 6

Anoxia-induced increases in intracellular calcium concentration in primary cultures of rabbit thick ascending limb of Henle's loop

U.M. Rose, A. Hartog, J.W.C.M. Jansen, C.H. Van Os and R.J.M. Bindels
in: Biochimica and Biophysica Acta (submitted)

ABSTRACT

The effect of anoxia on $[Ca^{2+}]_i$ in primary cultures of mTAL and cTAL was investigated. Previously, we reported a method to monitor $[Ca^{2+}]_i$ continuously in cultured PT cells during 1 h of anoxic incubation in the absence of glycolytic substrates (Rose et al., 1993). Complete absence of O_2 was realized by inclusion of a mixture of oxygenases in an anoxic chamber. As a result of substrate-free anoxia, $[Ca^{2+}]_i$ started to rise in individual cells of mTAL and cTAL monolayers and reached maximal levels within 60 min after starting the anoxic incubation. Anoxia induced significant increases in $[Ca^{2+}]_i$ from 76 ± 1 to 469 ± 18 nM in mTAL monolayers and from 58 ± 1 to 442 ± 27 nM in cTAL monolayers ($P < 0.05$). At the re–introduction of oxygen and glucose, elevated $[Ca^{2+}]_i$ rapidly declined to 110 ± 4 and 105 ± 5 nM in mTAL and cTAL, respectively ($P < 0.05$). Removal of Ca^{2+}_o and addition of 0.1 mM La^{3+} partially prevented anoxia-induced increases in $[Ca^{2+}]_i$ in both cell types. The L-type Ca^{2+} channel blocker D600 (1 μM) was as effective as Ca^{2+} removal and La^{3+} addition. Comparing mTAL and cTAL cells, only one difference was consistently observed. Prevention of Ca^{2+} influx by exposure to La^{3+} combined with Ca^{2+} removal or addition of 1 μM D600 had a greater inhibitory effect anoxic $[Ca^{2+}]_i$ values in mTAL than in cTAL monolayers, indicative for a larger role of Ca^{2+} influx through L-type Ca^{2+} channels in anoxia-induced increases in $[Ca^{2+}]_i$ in the former cell type. In conclusion, substrate-free anoxia reversibly increases $[Ca^{2+}]_i$ in primary cultures of cTAL and mTAL, which results from Ca^{2+} release from stores as well as from Ca^{2+} influx via D600-sensitive Ca^{2+} channels.

INTRODUCTION

Renal ischemia results in a variety of pathophysiological alterations at the level of the tubular epithelium, including impairment of intracellular Ca^{2+} homeostasis, perturbation of cytoskeletal organisation and activation of degradative enzymes (Brezis, 1993). The complex composition of the kidney, in terms of morphology and physiological and biochemical functions, necessitates detailed investigations of responses of the various nephron segments to ischemia. Distinct sensitivities to anoxia and hypoxia were observed in the epithelia from separate segments of the nephron (Wilson et al., 1986; Lash et al., 1990; Doctor et al., 1993). These different susceptibilities to anoxia can be attributed to hemodynamic factors as well as to biochemical or functional heterogeneity of the nephron (Weinberg, 1991). For instance, the differences in blood flow distribution between cortex and medulla could result in region specific cell damage in case of limited oxygen supply. The medulla, for example, has been shown to be especially sensitive for the development of injury during an ischemic insult (Wilson et al., 1986; Bonventre, 1993). Also, cell swelling or obstruction by cell debris, causing vascular congestion, can lead to abnormal differences in blood supply to the various kidney regions (Mason et al., 1989). Independent of these hemodynamic consequences, distinct intrinsic characteristics of the individual epithelial cell types may underlie the variable degree of sensitivity to ischemic injury along the nephron. In particular, a high degree of ischemic damage has been observed in the S_3 portion of the PT and in the mTAL compared to the other segments (Wilson et al., 1986; Lash et al., 1990).

The complexity of the kidney has severely hampered detailed studies on ischemic injury in the various nephron segments. This has prompted several investigators to use freshly-isolated and cultured tubular cells which for the first time enabled studies in which cell type specific effects of ischemia can be analyzed (Weinberg, 1991). An additional advantage of these *in vitro* models is that experiments can be performed in the absence of hemodynamic factors. Recently, we established a method to investigate increases in $[Ca^{2+}]_i$ in PTs in primary culture induced by anoxic periods up to 60 min (Rose et al., 1993). In that study complete absence of oxygen was achieved by inclusion of Oxyrase®, a mixture of oxygenases, to preclude any contribution of reactive oxygen species to cellular injury which may occur in model studies using chemical anoxia or

hypoxia (Weinberg, 1991). In the present study, we applied similar conditions to determine and compare the anoxia-induced increases in $[Ca^{2+}]_i$ in primary cultures of mTAL and cTAL to delineate biochemical and physiological mechanisms which may explain cell type specific sensitivities to anoxia.

MATERIALS AND METHODS

Isolation of kidney epithelial cells

Rabbit mTAL and cTAL cells were isolated by immunodissection as described previously for PT cells (Rose et al., 1993). Briefly, kidneys were excised from New Zealand white rabbits (≈0.5 kg). A cell suspension was obtained by enzymatic digestion of dissected medullary or cortical tissue to obtain mTAL and cTAL cells, respectively. The cell suspension was incubated for 60 min on ice with monoclonal antibody C109E11, recognizing TAL cell surface specific antigens. After three washings, the cell suspension was added to goat anti-mouse IgG-coated petri dishes. After 15 min of incubation at room temperature, the dishes were washed carefully and adherent cells were scraped off the dishes. The isolated mTAL or cTAL cells were collected and seeded at a density of $5x10^5$ cells/cm^2 on collagen-coated round coverslips (Ø 22 mm; Menzel, Germany) or on collagen-coated 24- or 96-well plates (Costar, Badhoevedorp, The Netherlands). Cells were grown to subconfluency in a mixture of Dulbecco's Modified Eagles medium (Imperial #1-466-14, Hampshire, UK) Ham's F12 medium (Gibco, #041-01765M, Paisley, UK) (1:1), supplemented with 5% (v/v) FCS, gentamycin (10 µg/ml), $NaHCO_3$ (25 mM), glutamine (14 mM), insulin (5 µg/ml), transferrin (5 µg/ml), hydrocortisone (50 nM), 0.5% (v/v) non-essential amino acids (Gibco, #043-01140H, Paisley, UK), prostaglandin E_1 (70 ng/ml), triiodothyronine (5 pM), Na_2SeO_3 (50 nM), pH 7.4; hereafter this culture medium is referred to as K_1 + 5% FCS medium. The mTAL and cTAL cells were incubated in a humidified incubator, gassed with 5% CO_2 in air, at 37 °C. The cells were used 48 h after seeding.

Characterization of mTAL and cTAL in primary culture

Hormone-induced cAMP formation was measured to characterize the primary cultures. mTAL and cTAL cells cultured on 96-well plates were washed twice with Krebs-Henseleit buffer (KHB; composition in mM: 128 NaCl, 5 KCl, 2 $CaCl_2$, 10 glucose, 10 Na-acetate, 4 l-lactate, 1 l-alanine, 20 HEPES/Tris, pH 7.4) and incubated at 37 °C with 100 µl/well 10^{-7} M bovine parathyroid hormone (bPTH, 1-34), calcitonin, vasopressin (AVP) or prostaglandin E_2 (PGE_2) in KHB containing 1 mM IBMX. After 10 min, the reaction was stopped by aspiration of the buffer and addition of 50 µl 0.2 N HCL to each well. The cAMP concentration was determined according to Brown et al. (1971).

The Na^+-K^+-2Cl- cotransport activity was determined by measuring bumetanide-

sensitive $^{86}Rb^+$ uptake in the presence of ouabain to inhibit the Na^+-K^+ pump All incubations were performed at 37 °C. Medium from the 24-well plates was aspirated and cells were preincubated for 5 min in 0 2 ml KHB containing 1 mM ouabain and in the presence or absence of 10 µM bumetanide (Leo Pharmaceutical Products, Ballerup, Denmark). Subsequently, the medium was replaced by 0.2 ml of the same KHB medium to which 0.5 µCi/ml $^{86}RbCl$ was added The $^{86}Rb^+$ uptake was stopped after 3 min by washing 4 times with 0.4 ml ice-cold KHB supplemented with 1 mM ouabain and 10 µM bumetanide. Plates were allowed to air dry and cells were subsequently lysed by adding 0.5 ml 0 05 % (v/v) sodium dodecyl sulphate to each well Radioactivity in the cell lysate was determined by liquid scintillation counting Protein concentration was determined with the Coomassie blue protein assay (Biorad, Richmond, CA, USA), using γ-immunoglobulin as a standard. In a separate series of experiments was determined that $^{86}Rb^+$ uptake was linear up to at least 5 min (data not shown).

Fura-2 loading

mTAL and cTAL cells were loaded with fura-2 by incubating coverslips with monolayers for 1 h at 37 °C in K_1 medium containing 5 µM fura-2 AM (Molecular Probes, Eugene, OR, USA), 0.02% (w/v) pluronic F127 (Molecular Probes, Eugene, OR, USA), 4% (v/v) FCS and 3 mM probenecid. After loading, the cells were washed twice in the experimental medium and used immediately All experiments were performed in the presence of 3 mM probenecid in order to inhibit fura-2 leakage via organic anion transporters.

Measuring $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by quantitative fluorescence microscopy using the Newcastle Photonic System (NPS, Newcastle, UK). This system contains a photomultiplier tube which is connected to a Nikon Diaphot inverted microscope with a 40x quartz oil immersion objective. The photometer contains a pin-hole diaphragm to determine the viewing field. In this study all data represent fluorescence measurements from 2 to 4 cells, captured at 400x magnification. The fura-2 loaded mTAL or cTAL cells were alternatingly excited at 340 and 380 nm and emitted light was collected at 1-s intervals at 510 nm

$[Ca^{2+}]_i$ was calculated according to the formula derived by Grynkiewicz et al (1985). $[Ca^{2+}]_i = K_D \times R_{bf} \times [(R-R_{min}) / (R_{max}-R)]$, where K_D is the dissociation constant of fura-2 for Ca^{2+} of 224 nM, R is the ratio of fluorescence of the cell at 340 and 380 nm, R_{max} and R_{min} represent the ratios of fura-2 fluorescence intensity at 340 and 380 nm excitation obtained by treating the cells with 5 µM ionomycin in the presence and absence (estimated by addition of 2 mM EGTA) of Ca^{2+}_o, respectively, R_{bf} is the maximal 380 nm signal divided by the minimal 380 nm signal. Most of the results, however, are presented as fura-2 fluorescence ratio values instead of real Ca^{2+} concentrations, since a calibration procedure could not be performed in every preparation because of experimental problems. Firstly, addition of the Ca^{2+} ionophore ionomycin led to abrupt rounding off of the cells and secondly, addition of

EGTA often caused detachment of the cells from the coverslip. These problems were also encountered in studies with cultured proximal tubule cells (Rose et al., 1993).

Anoxic chamber experiment

To estimate $[Ca^{2+}]_i$ during anoxia, an anoxic chamber was used as described previously (Rose et al., 1993). Briefly, fura-2 loaded mTAL or cTAL monolayers on a glass coverslip were mounted in an anoxic chamber at 37 °C. The chamber was filled with 100% N_2 gassed, modified KHB (composition in mM: 138 NaCl, 5 KCl, 5 l-lactate, 1 $MgSO_4$, 2 $CaCl_2$, 1 l-alanine, 20 HEPES/Tris and 360 mU/ml Oxyrase® (Oxyrase Inc., Ashland, Ohio, USA), pH 7.4). After mounting the chamber on the stage of the microscope, $[Ca^{2+}]_i$ was measured using the NPS. After 60 min of anoxia, reperfusion was started by perfusing the chamber with oxygenated KHB containing 10 mM glucose. In additional experiments cell viability was measured after 1 h of substrate-free anoxia in the anoxic chamber by means of LDH release into the incubation medium. LDH concentration was measured using a LDH assay described previously (Rose et al., 1993).

Materials

Collagenase A and hyaluronidase were obtained from Boehringer Mannheim (Germany). The Ca^{2+} channel blocker D600 was kindly provided by Knoll (Ludwigshaven, Germany). All other chemicals, hormones and conjugated antibodies were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified. All chemicals were of the purest grade available.

Statistical analysis

All reported data are expressed as means ± SE. Statistical analysis was performed on fura-2 ratio values using analysis of variance ($P < 0.05$ is significant). Subsequently, statistical significant differences between experimental groups were estimated by means of contrast analysis according to Fisher (Snedecor et al., 1974).

RESULTS

Characterization of primary cultures of mTAL and cTAL

Conventional immunoperoxidase staining with monoclonal antibody C109E11, confirmed that this antibody specifically recognizes mTAL (Fig. 1B, D) and cTAL (Fig. 1A, C). This antibody did not react with PTs, glomeruli, connecting tubules, distal convoluted tubules or collecting ducts. The immunodissected mTAL and cTAL cells were subsequently cultured on glass coverslips. The cultured cells reached 70 to 100% confluency within 48 h after seeding the cells and exhibited an epithelial-like appearance. All experiments were performed 2 days after seeding to prevent cellular de-differentiation which is a consequence of culturing. Table I shows the hormone-induced intracellular cAMP accumulation in cultured mTAL and cTAL. In both cultures, bPTH(1-34), calcitonin, AVP and PGE_2 stimulated cAMP production significantly. Subsequently, the presence of Na^+-K^+-2Cl- cotransport as indicated by bumetanide-sensitive ouabain-insensitive $^{86}Rb^+$ uptake was investigated in primary cultures of cTAL. In the presence of 1 mM ouabain, $^{86}Rb^+$ uptake amounted to 20 ± 2 $nmol.min^{-1}.mg\ protein^{-1}$ and 10^{-4} M bumetanide inhibited 69 ± 1% of this control uptake.

Table I. Hormone-induced cAMP formation in primary cultures of mTAL and cTAL

Hormone	cAMP (nmol · mg protein^{-1} ·10 min^{-1})	
	mTAL	cTAL
control	52 ± 13	31 ± 7
bPTH (1-34)	186 ± 53*	78 ± 22*
calcitonin	821 ± 104*	552 ± 85*
AVP	290 ± 75*	284 ± 89*
PGE_2	155 ± 29*	411 ± 131*

Values are mean ± SE with N ≥ 5. In all experiments, hormones were applied at a concentration of 10^{-7} M. *P < 0.05, significantly different from control.

$[Ca^{2+}]_i$ during anoxia

As demonstrated previously, oxygen was totally eliminated from the anoxic chamber within 10 min after filling the chamber with hypoxic medium containing the enzyme complex Oxyrase® (Rose et al., 1993). As a result of this substrate-free anoxia, $[Ca^{2+}]_i$ started to rise in individual cells of mTAL and cTAL monolayers and reached maximal levels within 60 min after starting the

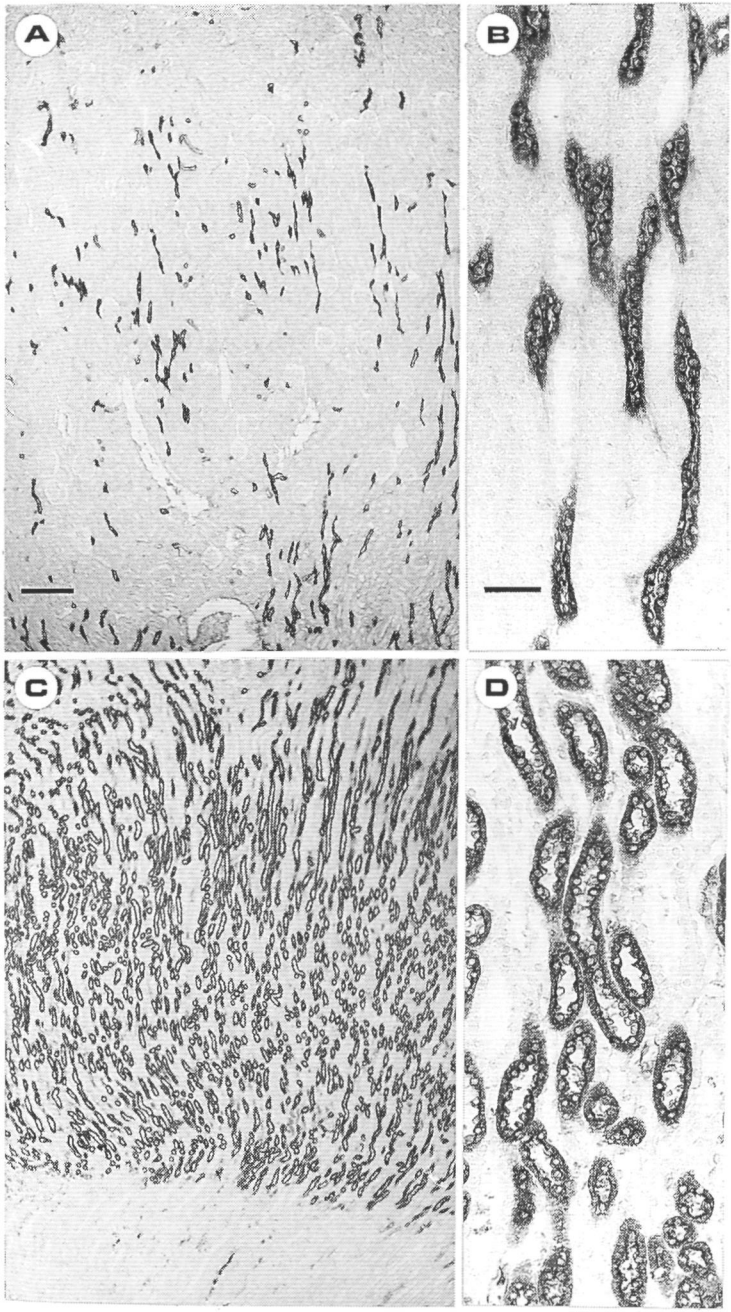


Figure 1
Immunoperoxidase staining with monoclonal antibody C109E11 of paraffin embedded cortical (**A, C**) or medullary (**B, D**) sections of rabbit kidney. In both sections, only thick ascending loop of Henle cells are positively stained. The bar represents 200 μm for figures A and B, and 50 μM for figures C and D.

measurements (Fig. 2). At the introduction of oxygen and glucose, i.e. reperfusion, the elevated fura-2 ratio declined rapidly towards pre-anoxic levels. As shown in figure 3, the maximal levels attained varied strongly between cells on one coverslip and between subsequent preparations. On average, the fura-2 fluorescence ratio increased significantly from 1.14 ± 0.01 to 2.33 ± 0.09 in mTAL and from 1.07 ± 0.02 to 2.27 ± 0.14 in cTAL ($P < 0.05$). After the introduction of oxygen and glucose the elevated fura-2 ratio declined within 10 min to 1.27 ± 0.04 and 1.25 ± 0.06 in mTAL and cTAL, respectively. These

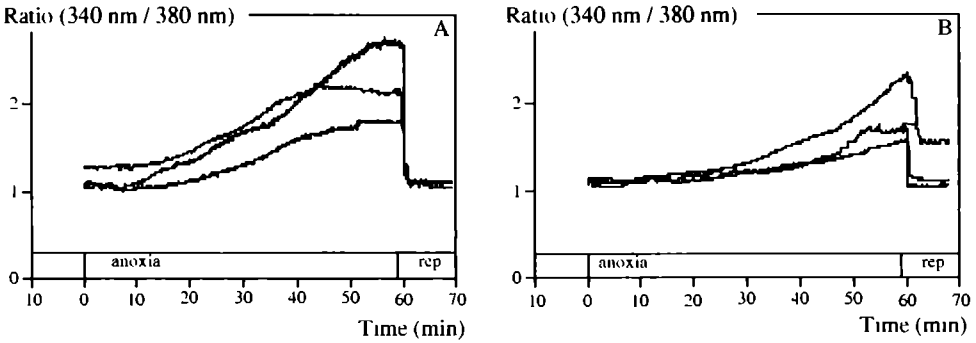


Figure 2

Time-dependency of increase in $[Ca^{2+}]_i$ in mTAL (A) and cTAL (B) cells in primary culture in response to substrate-free anoxia at 37 °C. At -10 min the anoxic chamber is closed and $[Ca^{2+}]_i$ measurements start at 0 min (anoxia). After 60 min of anoxia, oxygen and glucose are re-introduced (reperfusion: rep). $[Ca^{2+}]_i$ is presented as the 340 and 380 nm ratio of fura-2 excitation. Three typical experiments observed in 2-4 cells are shown.

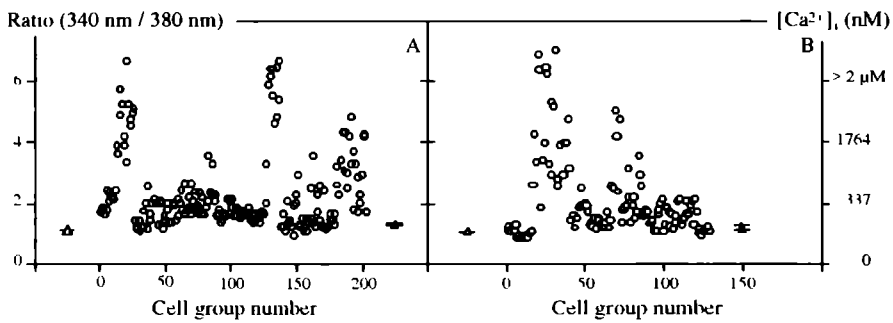


Figure 3

Summary of heterogeneous responses in $[Ca^{2+}]_i$ of primary cultures of mTAL (A) and cTAL (B) cells to substrate-free anoxia: Δ, mean basal $[Ca^{2+}]_i$ measured immediately after mounting the anoxic chamber on the stage of the microscope (10 min after filling the anoxic chamber); o, maximal $[Ca^{2+}]_i$ in cell groups reached after 60 min of anoxia; ▲, mean $[Ca^{2+}]_i$ reached 10 min after reperfusion with oxygenated glucose-containing buffer. $[Ca^{2+}]_i$ is presented as fura-2 ratio (left) and in nM (right).

reperfusion values were not significantly different from the basal values ($P > 0.1$), indicating that no irreversible damage occurred during the anoxic period. In addition, after 1 h of anoxia LDH release did not increase above 5% in both mTAL and cTAL cells. Moreover, no abrupt fura-2 loss occurred during 60 min of anoxia in both cell types, an indication that cell membrane barrier properties remained intact. Comparing the effects of anoxia on $[Ca^{2+}]_i$ in mTAL and cTAL, no significant differences in maximal anoxic nor reperfusion levels were observed ($P > 0.1$). The maximal $[Ca^{2+}]_i$ reached during anoxia minus the basal $[Ca^{2+}]_i$ was similar in mTAL and cTAL: 1.19 ± 0.10 and 1.31 ± 0.13, respectively ($P > 0.1$). Only,the basal $[Ca^{2+}]_i$ was significantly lower in cTAL than in mTAL ($P < 0.05$). In table II the mean values are given for all $[Ca^{2+}]_i$ values observed in both preparations.

Table II. Anoxia-induced increases in $[Ca^{2+}]_i$ in primary cultures of mTAL and cTAL

	$[Ca^{2+}]_i$ (nM)	
	mTAL	cTAL
Basal	76 ± 1	58 ± 1#
Maximal anoxic	469 ± 18*	442 ± 27*
Reperfusion	109 ± 4	105 ± 5
Δ	393 ± 15*	384 ± 23*

Anoxic incubation of mTAL and cTAL in primary culture. Basal, maximal anoxic and reperfusion $[Ca^{2+}]_i$, and Δ (maximal anoxic minus basal $[Ca^{2+}]_i$) are presented as calculated Ca^{2+} values in nM. All data are the mean ± SE of at least 38 cell groups from ≥ 4 separate cell preparations (*$P < 0.05$: compared with basal $[Ca^{2+}]_i$; #$P < 0.05$: mTAL versus cTAL).

Next, the dependence of anoxia-induced increases in $[Ca^{2+}]_i$ on $Ca^{2+}{}_o$ was studied by preventing Ca^{2+} influx and the results are shown in figure 4. Omitting Ca^{2+} from the medium resulted in a $[Ca^{2+}]_o$ of ≈20 μM and therefore 0.1 mM La^{3+} was added to block any residual Ca^{2+} influx. Blocking Ca^{2+} influx slightly reduced the basal fura-2 ratio, albeit significantly, and partially prevented anoxia-induced increases in fluorescence ratio in both cell types ($P < 0.05$). This indicates that the anoxia-induced rise in $[Ca^{2+}]_i$ is dependent on Ca^{2+} influx as well as on release from intracellular Ca^{2+} stores. Surprisingly, prevention of Ca^{2+} influx had a greater inhibitory effect on maximal anoxic and reperfusion

fura-2 ratio, and differences between basal and anoxic ratios in mTAL than in cTAL ($P < 0.05$). This points to a larger role of Ca^{2+}_o in anoxia-induced increases in $[Ca^{2+}]_i$ in the mTAL cell type.

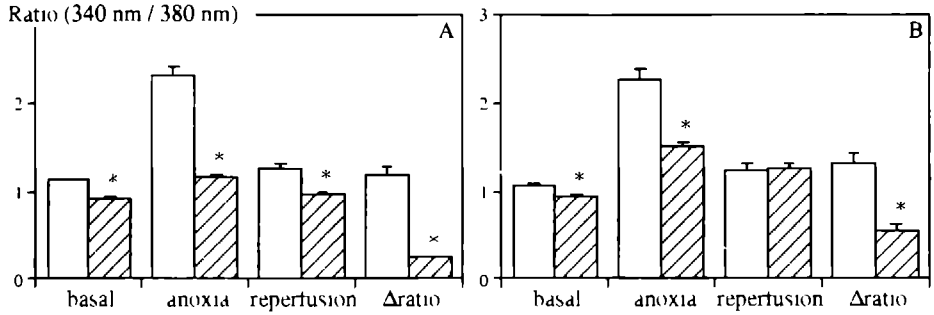


Figure 4

The effect of nominally Ca^{2+}-free solution plus 0.1 mM $LaCl_3$ (-Ca/+La) on basal, maximal anoxic and reperfusion fura-2 ratio values, and on Δratio values (i.e. maximal anoxic minus basal ratios) in primary cultures of mTAL (**A**) and cTAL (**B**). Fura-2 ratios observed in the Ca^{2+}-free situation (dashed columns) are compared to fura-2 ratios observed during control anoxic incubations in the presence of 2 mM $CaCl_2$ (open columns). Columns represent mean fura-2 ratio values ± SE with $N \geq 64$ (*$P < 0.05$: -Ca/+La versus control ratio values).

Since L-type Ca^{2+} channel blockers such as verapamil have been shown to reduce *in vivo* ischemic renal injury (Humes, 1986; Schrier et al., 1987; Cotterill et al., 1989), and since D600 also reduced anoxia-induced increases in $[Ca^{2+}]_i$ in primary cultures of PTs (Rose et al., 1993), the effect of D600 on anoxia-induced increases in $[Ca^{2+}]_i$ in mTAL and cTAL was investigated. Figure 5 demonstrates that in both cell types 1 μM D600 significantly reduced the maximal $[Ca^{2+}]_i$ reached during anoxia. A reduction in the difference between basal and anoxic fura-2 ratio was observed, while no effect was seen on basal $[Ca^{2+}]_i$. After reperfusion, $[Ca^{2+}]_i$ rapidly declined and stabilized in the presence of D600 at basal levels. When the effects of D600 on mTAL and cTAL are compared, it is evident that 1 μM D600 has a larger inhibitory effect on reperfusion $[Ca^{2+}]_i$ and on the difference between basal and anoxic fura-2 ratio in mTAL than in cTAL ($P < 0.05$), which confirms that anoxia-mediated Ca^{2+} influx is more substantial in mTAL than in cTAL.

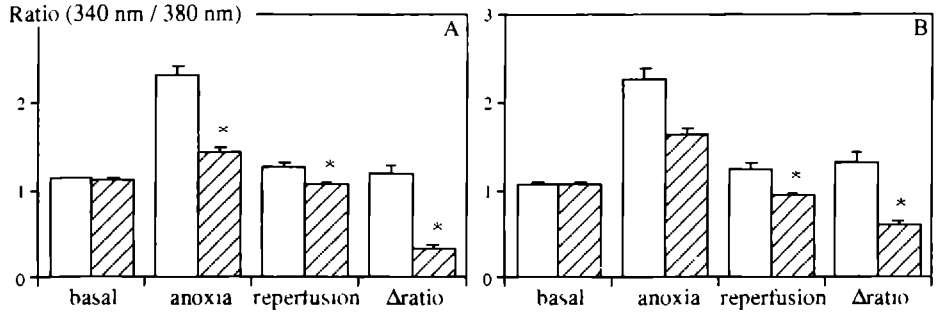


Figure 5

The effect of 1 μM D600 on basal, maximal anoxic and reperfusion fura-2 ratio values, and on Δratio values (i.e. maximal anoxic minus basal ratios) in primary cultures of mTAL (**A**) and cTAL (**B**). Fura-2 ratios observed in the presence of D600 (dashed columns) are compared to ratios observed in the absence of D600 (open columns). Columns represent mean fura-2 ratio values ± SE with N ≥ 50 (*P < 0.05: D600 versus control ratios).

DISCUSSION

The present study demonstrates that substrate-free anoxia increases $[Ca^{2+}]_i$ in mTAL and cTAL cells in primary culture. In addition, the L-type Ca^{2+} channel blocker D600 and exclusion of $Ca^{2+}{}_o$ only partly reduced elevated levels of $[Ca^{2+}]_i$ in these monolayers. This suggests that in both cell types Ca^{2+} influx via L-type Ca^{2+} channels as well as Ca^{2+} release from intracellular stores contribute to anoxia-induced increases in $[Ca^{2+}]_i$.

The primary cultures originate from immunodissected mTAL and cTAL cells using a monoclonal antibody that recognized plasma membrane specific antigens. In this way, only mTAL and cTAL cells were isolated from rabbit kidney medullary and cortical cell suspensions, respectively. Recently, we reported that this technique yielded monolayers of PTs or cortical collecting system that retained several characteristics of the original nephron segment (Bindels et al., 1991; Rose et al., 1993). In the present study, mTAL and cTAL cells in primary culture accumulated cAMP when treated with bPTH, AVP, PGE_2 or calcitonin. This pattern of hormonal responses is similar to those reported previously with freshly-isolated mTAL and cTAL segments, except for PTH receptors which were only present in freshly-isolated cTAL (Morel, 1981; Nakao et al., 1989). The cTAL cultures exhibited Na^+-K^+-2Cl- cotransport as indicated by bumetanide-sensitive ouabain-insensitive $^{86}Rb^+$ uptake. These

findings demonstrate that the primary cultures retained functions which are typical for the thick ascending limb of Henle's loop.

Previously, we established an *in vitro* model system to study changes in $[Ca^{2+}]_i$ in single cultured renal cells during prolonged periods of anoxia (Rose et al., 1993). Complete anoxia, i.e. the partial O_2 pressure is 0 mmHg, was established by adding an oxygenase mixture to N_2-gassed medium. However, anoxia-induced increases in $[Ca^{2+}]_i$ were only apparent in the absence of glucose and acetate. When these conditions, i.e. absence of oxygen and substrates for glycolysis, were applied to mTAL and cTAL cells in primary culture elevated levels of $[Ca^{2+}]_i$ were attained which were heterogeneous with respect to the onset as well as to the steady-state level reached. Nevertheless, in mTAL and cTAL monolayers similar maximal $[Ca^{2+}]_i$ were achieved within 60 min of anoxia, with a mean value of 469 ± 18 and 442 ± 27 nM, respectively. These anoxic Ca^{2+} levels remained stable until oxygen was re-introduced. Considering the large electrochemical gradient favouring Ca^{2+} influx, the increases in $[Ca^{2+}]_i$ are surprisingly modest. These observations are in line with previous studies on anoxia-induced cellular injury in freshly-isolated and cultured kidney cells where similar small increases in $[Ca^{2+}]_i$ were reported (Rose et al., 1993). Additionally, others reported increases in $[Ca^{2+}]_i$ after metabolic inhibition in cultured PT cells (McCoy et al., 1988; Phelps et al., 1989). The anoxia-induced increases in $[Ca^{2+}]_i$ in mTAL and cTAL cells were reversible on re-oxygenation since reperfusion rapidly restored $[Ca^{2+}]_i$ to pre-anoxic levels. This finding indicates that the cultured cells remain viable during 60 min of anoxia, which is confirmed by the absence of cellular LDH or sudden fura-2 loss as a sign of cell death. Apparently, the rise in $[Ca^{2+}]_i$ is an early event in anoxia-mediated disturbances which leads eventually to cell injury or, alternatively, Ca^{2+} is not a primary mediator of cellular damage associated with ischemia.

Increased $[Ca^{2+}]_i$ can be involved in a number of processes that are detrimental to the cell, as several Ca^{2+}-dependent compounds of cellular injury have been appreciated including disintegration of brush-borders, mitochondria and cytoskeletal organization (Brezis, 1993). Of interest is the recent observation by Doctor et al. (1993) that renal ischemia induces cTAL cells to specifically and extensively degrade ankyrin, a pivotal protein in the spectrin-connected cytoskeleton. These authors hypothesized that anoxia-mediated increases in $[Ca^{2+}]_i$ activate Ca^{2+}-dependent proteases, such as calpain which in

turn are capable to degrade proteolytically a variety of different proteins including cytoskeletal proteins. Also in cultured renal cells, activation of calpain has been correlated with molecular mechanisms involved in renal cell injury (Wilson et al., 1986; Brezis, 1993). Future studies are awaited to delineate the detrimental role of ischemia-induced proteolysis of cytoskeleton-related proteins in TAL.

In general, an important role for increased Ca^{2+} influx is implicated in the pathogenesis of ischemic renal injury (Wilson et al., 1986; Bonventre, 1993). For instance, lowering $[Ca^{2+}]_o$ prevented effectively the development of hypoxic TAL necrosis in the isolated perfused rat kidney (Shanley et al., 1991). Therefore, manoeuvres were designed to prevent Ca^{2+} entry into the cell, all of which reduced anoxia-mediated $[Ca^{2+}]_i$ increase. D600 was as effective as removal of $Ca^{2+}{}_o$ and addition of La^{3+}, which identifies L-type Ca^{2+} channels as anoxia-mediated Ca^{2+} entry pathway. Evidence for the presence of such channels in TAL originates from previous studies in cultures of a mixture of cells from TAL and distal convoluted tubules (Bacskai et al., 1990). Using these cultures, Bacskai and Friedman demonstrated that PTH-induced increases in $[Ca^{2+}]_i$ were significantly inhibited by Ca^{2+} channel blockers while pre-stimulated Ca^{2+} levels, like in the present study, were not influenced. Under steady-state conditions, these Ca^{2+} channels are apparently functionally absent and during anoxia or hormonal stimulation they become activated.

Our observation that the increase in $[Ca^{2+}]_i$ was only partly abolished by removing $Ca^{2+}{}_o$ and adding La^{3+} or applying D600, suggests that besides Ca^{2+} influx also intracellular Ca^{2+} stores contribute to anoxia-induced increases in $[Ca^{2+}]_i$. Within the loop of Henle, the contribution of the Ca^{2+} influx component was most pronounced in mTAL compared to cTAL. These findings are in contrast with observations in freshly-isolated (Rose et al., 1994) and cultured PTs (Rose et al., 1993) where anoxia-induced increases in $[Ca^{2+}]_i$ completely depend on $Ca^{2+}{}_o$. Although this functional differences in response to anoxia between PTs and TAL did not lead to different susceptibilities to anoxia-induced cell injury, they may be responsible for the different susceptibilities to oxidative stress between both nephron segments as described by Lash et al. (1990). In addition, differences in sensitivity could also become apparent after longer anoxic incubations than 1 h.

From the present study it can not be concluded that intrinsic properties of

nephron segments account for different susceptibilities to hypoxia or ischemia as observed *in vivo* (Wilson et al., 1986; Bonventre, 1993), since primary renal cell cultures are equally sensitive to oxidative stress in terms of maximal levels of $[Ca^{2+}]_i$ attained during anoxia, reversibility of accumulated Ca^{2+} and absence of cellular injury. Consequently, hemodynamic factors might be more critical in ischemic related renal damage. Due to different levels of blood supply, for example, variable levels of hypoxia are obtained during ischemia (Kehrer et al., 1990). In this respect, it has been reported that the blood supply decreased more in the medullary than in the cortical region after ischemia, resulting in a higher risk of cell injury in the medulla (Bonventre, 1993). Unfortunately, cultured renal cells express a greater capability to counteract oxidative stress than freshly-isolated cells (Weinberg, 1991). Thus, the possibility can not be excluded that the decreased sensitivity to anoxia of cultured cells masks some functional aspects of nephron heterogeneity.

In conclusion, anoxia induced increases in $[Ca^{2+}]_i$ in primary cultures of TAL, which results from Ca^{2+} release from stores as well as from Ca^{2+} influx via D600-sensitive Ca^{2+} channels. However, during 60 min of anoxia these altered cellular functions were not accompanied by cytotoxic effects resulting in cell death.

CHAPTER 7

Effects of substrate-free anoxia and veratridine on intracellular calcium concentration in isolated rat ventricular cardiomyocytes

U.M. Rose, J.W.C.M. Jansen, R.J.M. Bindels and C.H. Van Os
in: Pflügers Archiv (in press)

ABSTRACT

Cytosolic free Ca^{2+} concentration was measured in freshly-isolated rat ventricular cardiomyocytes during substrate-free anoxia. Cardiomyocytes were loaded with fura-2 and incubated in an anoxic chamber in which PO_2=0 mmHg was realized by inclusion of Oxyrase®. $[Ca^{2+}]_i$ was measured in individual cells using digital imaging fluorescence microscopy. During anoxia, the shape of cardiomyocytes changed from a relaxed-elongated form into a rigor configuration within 15 min after the onset of anoxia. After the cells had developed the rigor state, a delayed rise in $[Ca^{2+}]_i$ reached a stable maximal level within 45 min. The mean values for the pre-anoxic and maximal anoxic $[Ca^{2+}]_i$ were 52 ± 3 nM (N = 42) and 2115 ± 59 nM (N = 45), respectively. The purported Na^+ overload blocker R 56865, significantly reduced maximal anoxic $[Ca^{2+}]_i$ to 553 ± 56 nM ($P < 0.05$), implicating a role of elevated intracellular Na^+ in anoxia-induced increases in $[Ca^{2+}]_i$. Veratridine (30 μM), which induces Na^+ overload, increased $[Ca^{2+}]_i$ to 787 ± 39 nM. The compound R 56865 reduced veratridine-induced increases in $[Ca^{2+}]_i$ to 152 ± 38 nM. Upon reperfusion, after 45 min of anoxia, two distinct responses were observed. Most often, $[Ca^{2+}]_i$ decreased upon reperfusion without a change in morphology or viability, while in the minority, $[Ca^{2+}]_i$ increased further which was followed by hypercontraction and loss of cell viability. The mean value for $[Ca^{2+}]_i$ 10 min after reperfusion of the former group, was 752 ± 46 nM (N = 38). The cardiomyocyte cell shape was followed by monitoring changes in the total fura-2 fluorescence (340 + 380 nm signal). Within 15 min after the onset of anoxia, the total fluorescence signal increased suddenly, before $[Ca^{2+}]_i$ started to rise, and this coincides with the onset of rigor contraction induced by ATP depletion.

INTRODUCTION

Cardiac ischemia is caused by reduced or absent coronary blood flow, leading to oxygen and metabolic substrate deprivation, which results in abnormal accumulation of ions in cardiac cells (Lee et al., 1991). In these pathological conditions, more Ca^{2+} crosses the sarcolemma than can be sequestered and extruded, causing Ca^{2+} overload (Ver Donck et al., 1991). As a result, pathological events as arrhythmias and mechanical dysfunction are triggered (Lee et al., 1991; Ver Donck et al., 1991). The effects of ischemia are time-dependent with a transition from reversible to irreversible changes in mechanical function (Tani et al., 1989), which can be reversed by timely reperfusion (Li et al., 1989). However, reperfusion can also cause additional damage, resulting in cell death of cardiomyocytes (Buja et al., 1988; Marsh et al., 1993a). As in the ischemic period, Ca^{2+} overload is also suggested to play a key role in the induction of reperfusion injury (Buja et al., 1988; Tani et al., 1989, 1990; Jeremy et al., 1992). Ca^{2+} overload during ischemia and/or reperfusion may involve a variety of mechanisms including Ca^{2+} influx via Ca^{2+} channels, Ca^{2+} release from intracellular stores such as the sarcoplasmic reticulum, or Ca^{2+} exchange against intracellularly accumulated Na^+ via reversed operation of the Na^+-Ca^{2+} exchanger. The role of Ca^{2+} channels, however, is equivocal because Ca^{2+} channel blockers have been shown to be protective in some studies (Haworth et al., 1987; Steenbergen et al., 1990; Hano et al., 1991), or have no protective effects against anoxia-induced cell injury (Hano et al., 1991) or reperfusion-induced Ca^{2+} overload (Watts et al., 1980). In addition, Ca^{2+} release from the sarcoplasmic reticulum would seem not to play a role in the induction of Ca^{2+} overload because pretreatment with thapsigargine, an inhibitor of the Ca^{2+}-ATPase in the sarcoplasmic reticulum could not prevent anoxia-induced increases in $[Ca^{2+}]_i$ (Miyata et al., 1992). Similar effects were found with caffeine (Allshire et al., 1987). Since Ca^{2+} influx through Ca^{2+} channels and Ca^{2+} release from stores are not involved in generating Ca^{2+} overload, the Na^+-Ca^{2+} exchanger seems to be the most likely mechanism. In this respect, hypoxia-induced increases in cytosolic Na^+ ($[Na^+]_i$) have been shown to result in Ca^{2+} overload by reversed operation of the Na^+-Ca^{2+} exchanger (Haigney et al., 1992).

In a previous anoxic study we demonstrated that anoxia induced increases in $[Ca^{2+}]_i$ in renal proximal tubule cells (Rose et al., 1993, 1994). However, the anoxic Ca^{2+} levels were surprisingly low in these non-excitable cells (Rose et al., 1993, 1994). In the present study, the effects of anoxia on proximal tubule cells and cardiomyocytes will be compared. In addition, anoxia-induced increases in $[Ca^{2+}]_i$ will be compared to the effects of veratridine on cardiomyocytes. Veratridine opens Na^+ channels, and is often used as a tool to induce Ca^{2+} overload by the Na^+-Ca^{2+} exchanger (Pauwels et al., 1989).

MATERIALS AND METHODS

Isolation of ventricular cardiomyocytes

A male Wistar rat weighing ≈0.5 kg, was anaesthetized with an intraperitoneal injection of 0.5 ml Nembutal® (Rousselot, Paris, France). The animal was heparinized intravenously (2000 U/kg) and the heart was rapidly excised. Cardiomyocytes were isolated according to the method described by ter Welle et al. (1988) with some minor modifications. Firstly, the heart was retrogradely perfused via the aorta with normal Ca^{2+} Krebs-Henseleit buffer (KHB; composition in mM: 3.3 KCl, 4.3 $NaHCO_3$, 1.4 KH_2PO_4, 2.0 $MgCl_2$, 155 NaCl, 11.1 glucose, 1.5 $CaCl_2$, 16.8 HEPES/NaOH, pH 7.35) gassed with 95% O_2 / 5% CO_2 and warmed to 37 °C. The perfusion pressure was 50 mmHg. After 15 min, the perfusate was changed to a low Ca^{2+} KHB (≈20 µM Ca^{2+}). After 5 min of perfusion with low Ca^{2+} KHB perfusion, the heart was perfused with low Ca^{2+} KHB supplemented with 0.1% collagenase B (w/v) (Boehringer, Mannheim, Germany). After perfusion of 100 ml, the perfusion-medium is changed to low Ca^{2+} KHB containing 0.05 % (w/v) collagenase B. When 100 ml perfusate is collected and the perfusion pressure has fallen to near zero, the heart is removed. The ventricles are cut into pieces and are dissociated in low Ca^{2+} KHB supplemented with 0.05% collagenase B and 2% (w/v) fatty acid free BSA (Boehringer, Mannheim, Germany). After 15 min, the dispersed cells are collected and the remaining tissue is again incubated for another 15 min. This procedure is repeated until no more viable cells are collected. The collected cells are centrifuged at 200 x g for 2 min, resuspended in low Ca^{2+} buffer containing 1% (w/v) BSA and kept at 37 °C until use. For experimental use a fraction of the cardiomyocyte suspension is washed in BSA-free low Ca^{2+} medium.

Fura-2 loading of ventricular cardiomyocytes

Cardiomyocytes in low Ca^{2+} medium are attached to poly-l-lysin coated round coverslips (Ø 22 mm; Menzel, Germany) at high density: 1 ml poly-l-lysine (Sigma, St. Louis, MO, USA) was brought onto the glass coverslip and incubated for 30 min at 20 °C. Thereafter, the

coverslip was washed with MilliQ® (Millipore) water Cells were brought onto and attached to the coverslip within 15 min at 37 °C in KHB medium. Non-attached cells were removed and attached cells were loaded with fura-2 by incubating the coverslip with cardiomyocytes for 1 h at 37 °C in low Ca^{2+} KHB, supplemented with 1% BSA, containing 5 µM fura-2 AM (Molecular Probes, Eugene, OR, USA), 0 02% (w/v) pluronic F127 (Molecular Probes, Eugene, OR, USA), 4% (v/v) FCS After loading, the cardiomyocytes were washed twice in the glucose-free modified KHB (for composition see under 'Anoxic chamber experiment') and were used immediately.

Measuring $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in single cardiomyocytes was measured by quantitative fluorescence microscopy using the MagiCal system (Applied Imaging Techniques, Tyne & Wear, UK). The fura-2-loaded cardiomyocytes were alternatingly excited at 340 and 380 nm and emitted light was captured at 510 nm with a CCD camera followed by digital imaging using TARDIS® software (Applied Imaging Techniques, Tyne & Wear, UK). The 340-380 nm capturing sequence was interrupted by 30 s of no capturing, divided into 10 s excitation at 340 nm allowing for cell focusing, and 20 s of no excitation using a shutter to avoid bleaching. $[Ca^{2+}]_i$ was calculated according to the formula derived by Grynkiewicz et al.(1985): $[Ca^{2+}]_i = K_D \times R_{bf} \times [(R-R_{min}) / (R_{max}-R)]$, where K_D is the dissociation constant of fura-2 for Ca^{2+} of 224 nM; R is the ratio of fluorescence in the cell at 340 and 380 nm; R_{max} and R_{min} represent the ratios of fura-2 fluorescence intensity at 340 and 380 nm excitation obtained by treating the cells with 4 µM ionomycin in the presence, R_{min} and absence, R_{max}, of 4 mM EGTA, respectively; R_{bf} is the maximal 380 nm signal divided by the minimal 380 nm signal

Measurements of changes in cell shape

Changes in cell shape were monitored as described in detail by Muallem et al (1992). Briefly, fura-2-loaded cardiomyocytes were alternatingly excited at 340 and 380 nm, and emitted light was captured at 510 nm with a CCD camera. After the experiment, total fluorescence was estimated by summation of the 340 and 380 nm signal A change in total fluorescence is a marker for a change in cell shape or volume, i.e. an increase in fluorescence is interpreted as an increase in dye concentration due to a decrease in cell volume (Muallem et al., 1992; Tauc et al., 1990) or, alternatively, a change in the optical geometry due to contracture.

Anoxic chamber experiment

To estimate $[Ca^{2+}]_i$ during anoxia, an anoxic chamber was used which has previously been described (Rose et al., 1993). Briefly, fura-2 loaded cardiomyocytes on a coverslip were mounted in an anoxic chamber at 37 °C. After filling the anoxic chamber with 100% N_2 gassed glucose-free modified KHB (composition in mM: 138 NaCl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1 l-alanine, 5 l-lactate, 20 HEPES/Tris) and 360 mU/ml Oxyrase® (Oxyrase Inc.,

Ashland, Ohio, USA), which removes traces of O_2 enzymatically, pH 7.4), $[Ca^{2+}]_i$ was measured using the MagiCal system. Cell viability was estimated by trypan blue exclusion. Cardiomyocytes were incubated for 1-2 min in 0.08% (w/v) trypan blue and the percentage stained cells was determined using light microscopy.

Materials

R 56865 was obtained from Solvay Duphar B.V. (Weesp, The Netherlands), veratridine from Sigma (St. Louis, MO, USA). All other chemicals used were of the purest grade available.

Statistical analysis

All reported data are expressed as means ± SE. Statistical analysis was performed on ratio values using analysis of variance ($P < 0.05$ is significant). Subsequently, statistical differences between experimental groups were estimated by means of contrast analysis according to Fisher (Snedecor et al., 1974).

RESULTS

Characterization of the freshly-isolated ventricular cardiomyocytes

After isolation, cardiomyocytes had a rod-shaped appearance with cross striations. These rod-shaped cells were quiescent in 2 mM $CaCl_2$ medium. The cell suspension also contained contracting cardiomyocytes which developed hypercontraction and soon lost viability. Also some hypercontracted, which are non-viable cells were present immediately after the isolation. Viable as well as non-viable cardiomyocytes attached to the poly-l-lysine coated coverslips. For $[Ca^{2+}]_i$ measurements, however, only viable cells were monitored, since cells with severely damaged cell membranes have lost their fura-2 content. The isolated cardiomyocyte suspension was kept for maximally 6 h following the isolation in oxygenated low Ca^{2+} KHB at 37 °C.

$[Ca^{2+}]_i$ during substrate-free anoxia

Under normoxic conditions, $[Ca^{2+}]_i$ of the cardiomyocytes was 52 ± 3 nM. Figures 1A and 2A show the response of single cardiomyocytes to substrate-free anoxic KHB. During anoxia, the initial $[Ca^{2+}]_i$ measurements start 10 min after closing the anoxic chamber, and $[Ca^{2+}]_i$ gradually increased from 52 ± 3 nM to a mean maximal $[Ca^{2+}]_i$ of 2115 ± 59 nM. This maximal Ca^{2+} level was stable until the re-introduction of glucose and oxygen. Upon reperfusion two types of

responses were observed. In most cardiomyocytes, the fura-2 ratio declined upon reperfusion (Fig. 1A). The mean $[Ca^{2+}]_i$ 10 min after the onset of reperfusion was 752 ± 46 nM, which is significantly higher than the initial $[Ca^{2+}]_i$ ($P < 0.05$). In some cardiomyocytes, however, the fura-2 ratio even increased upon reperfusion (Fig. 2A) and eventually lost their fura-2 content, which is indicative of cell death (Rose et al., 1993, 1994). As figure 3 demonstrates, cardiomyocytes respond heterogeneously to anoxia since different maximal anoxic and reperfusion Ca^{2+} levels were reached in individual cells. Table I shows the average $[Ca^{2+}]_i$ values obtained after calibration in normoxic and anoxic conditions.

Hypoxic Ca^{2+} levels have been shown to be linked to a rise in $[Na^+]_i$ (Haigney et al., 1992). Therefore, the effect of R 56865, a purported Na^+ overload blocker, was studied on anoxia-induced increases in $[Ca^{2+}]_i$. R 56865 (1 μM) significantly reduced the maximal anoxic $[Ca^{2+}]_i$ from 2115 ± 59 nM to 553 ± 56 nM ($P < 0.05$; Fig. 4). In addition, after reperfusion $[Ca^{2+}]_i$ was significantly decreased from 752 ± 46 nM to 327 ± 56 nM ($P < 0.05$) due to the presence of R 56865.

Table I. Intracellular Ca^{2+} concentration in ventricular cardiomyocytes

Condition	Anoxic chamber		Condition	Veratridine (30 μM)	
	control (nM)	+R 56865 (nM)		control (nM)	+R 56865 (nM)
normoxia	52 ± 3	68 ± 4	initial	37 ± 2	34 ± 3
anoxia	2115 ± 59*	553 ± 56*#	vrt	787 ± 39*	152 ± 38#
reperfusion	752 ± 46*	327 ± 56*#			

$[Ca^{2+}]_i$ of cardiomyocytes measured in an anoxic chamber compared with $[Ca^{2+}]_i$ resulting from incubation with 30 μM veratridine (vrt) at normoxic conditions. Values obtained in the anoxic chamber are measured initially (normoxia), at the maximum of anoxia-induced increases (anoxia) and 10 min after reperfusion. The $[Ca^{2+}]_i$ in the veratridine column are measured initially in nominally Ca^{2+} free solution ($[Ca^{2+}]_o \approx 20$ μM: initial) and 5 min after addition of 30 μM veratridine in the presence of 2 mM $CaCl_2$ in the bathing solution (vrt). The anoxic chamber and veratridine experiments were performed in the absence (control) and the presence (+ R 56865) of R 56865 (1 μM for the anoxic chamber and 10 μM for the veratridine experiment). Mean values ± SE are presented with $N \geq 25$ for the anoxic chamber experiments and $N \geq 6$ for the veratridine experiments (* $P < 0.05$: anoxia versus normoxia, reperfusion versus normoxia and veratridine versus initial; #$P < 0.05$: control versus + R 56865).

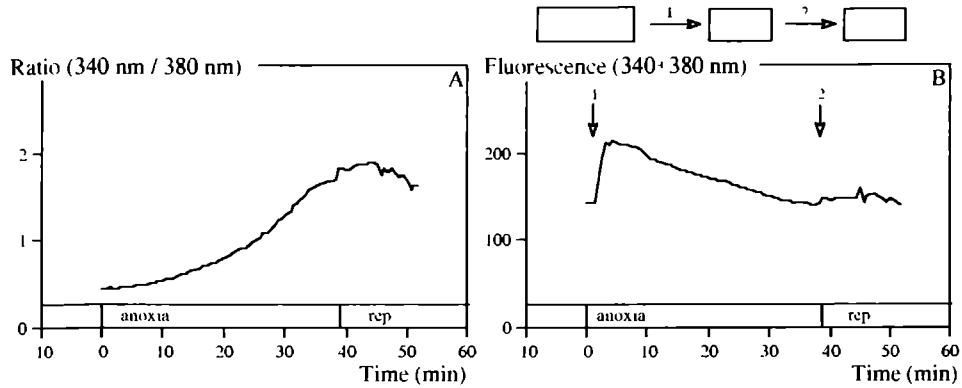


Figure 1

Anoxia-induced changes in $[Ca^{2+}]_i$ (**A**) and in cell shape (**B**) of a ventricular cardiomyocyte. **A.** Typical $[Ca^{2+}]_i$ measurement during anoxia, followed by reperfusion (rep). $[Ca^{2+}]_i$ is presented as the fura-2 ratio in one single cardiomyocyte as measured with the MagiCal system. **B.** Typical change in the shape of the same individual cardiomyocyte resulting from anoxia and reperfusion. The cardiomyocyte contracted during anoxia (1) to the rigor state resulting in an increase in total fluorescence (340+380 nm). At reperfusion, the cardiomyocyte remained in the rigor state, and no further change in cell shape occurred (2). Cell shape is monitored as the total fura-2 fluorescence: an increase in total fluorescence corresponds to contraction and change in optical geometry in cell volume.

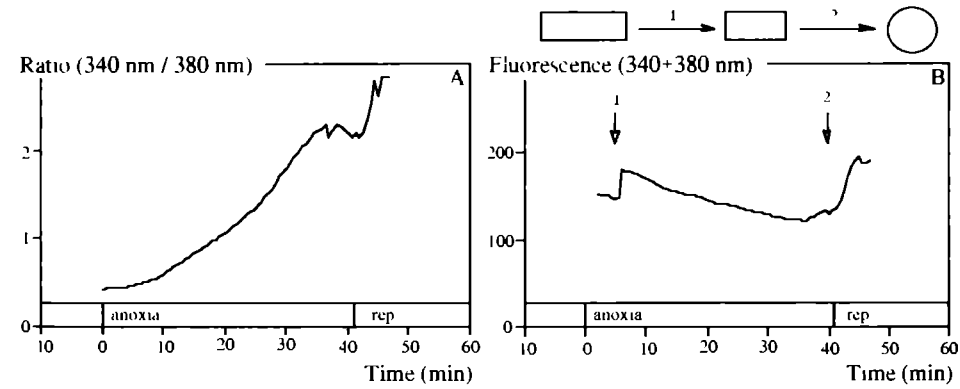


Figure 2

Anoxia-induced changes in $[Ca^{2+}]_i$ (**A**) and in cell shape (**B**) of a ventricular cardiomyocyte. **A.** Typical $[Ca^{2+}]_i$ measurement during anoxia, followed by reperfusion (rep). $[Ca^{2+}]_i$ is presented as the fura-2 ratio in one single cardiomyocyte as measured with the MagiCal system. **B.** Typical change in shape of the same individual cardiomyocyte resulting from anoxia and reperfusion. The cardiomyocyte contracted during anoxia (1) to the rigor state resulting in an increase in total fluorescence (340+380 nm)). At reperfusion, the cardiomyocyte develops hypercontraction, resulting in a further increase in fluorescence (2). Cell shape is monitored as the total fura-2 fluorescence: an increase in total fluorescence corresponds to contraction and change in optical geometry in cell volume.

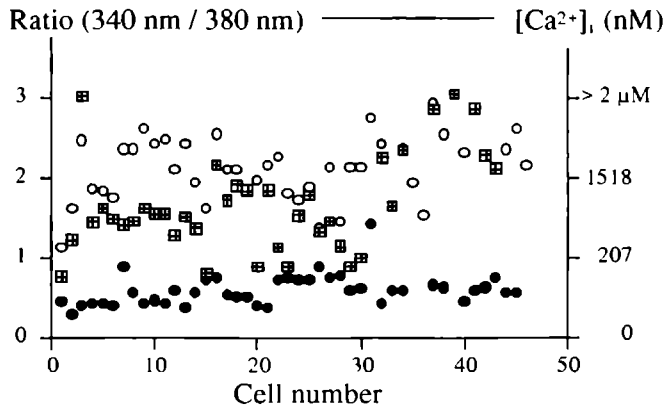


Figure 3

Heterogeneous responses of cardiomyocytes to anoxic incubation: ●, initial $[Ca^{2+}]_i$ measured immediately after filling the anoxic chamber; o, maximal anoxic $[Ca^{2+}]_i$, and ⊞, $[Ca^{2+}]_i$ reached upon reperfusion. $[Ca^{2+}]_i$ is presented as fura-2 ratio (left) or as Ca^{2+} concentration (right). Each point represents the fura-2 ratio or the $[Ca^{2+}]_i$ value observed in one single cell using the MagiCal system.

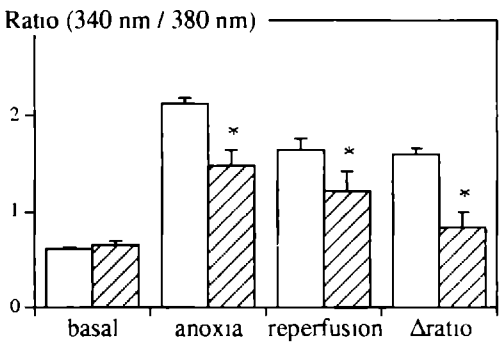


Figure 4

The effect of 1 µM R 56865 on initial (basal), maximal anoxic (anoxia), and reperfusion fura-2 ratio values and on Δratio values (i.e. maximal minus initial ratio values). Ratios observed in the presence of R 56865 (dashed columns) are compared to ratios observed in the absence of R 56865 (open columns). Columns represent mean ratios ± SE with N ≥ 25 (*P < 0.05: R 56865 versus control ratios).

Morphology during anoxia

In addition to measuring $[Ca^{2+}]_i$, the cell shape was monitored during the substrate-free anoxic incubation. Initially, fura-2 containing cardiomyocytes were rod-shaped with cross striations as described for the cells directly after the isolation procedure. Within 15 min after closing the anoxic chamber, the cardiomyocytes developed a contracture (Fig. 5), which is indicative of ATP depletion (Haigney et al., 1992; Bowers et al., 1993). During this initial period, $[Ca^{2+}]_i$ did not increase. During the subsequent anoxic period $[Ca^{2+}]_i$ gradually increased, but no further change in cell morphology was observed. Upon reperfusion, most cells remained in rigor contracture. Some cells, however, developed a hypercontractive state with further shortening and loss of sarcomere pattern. In

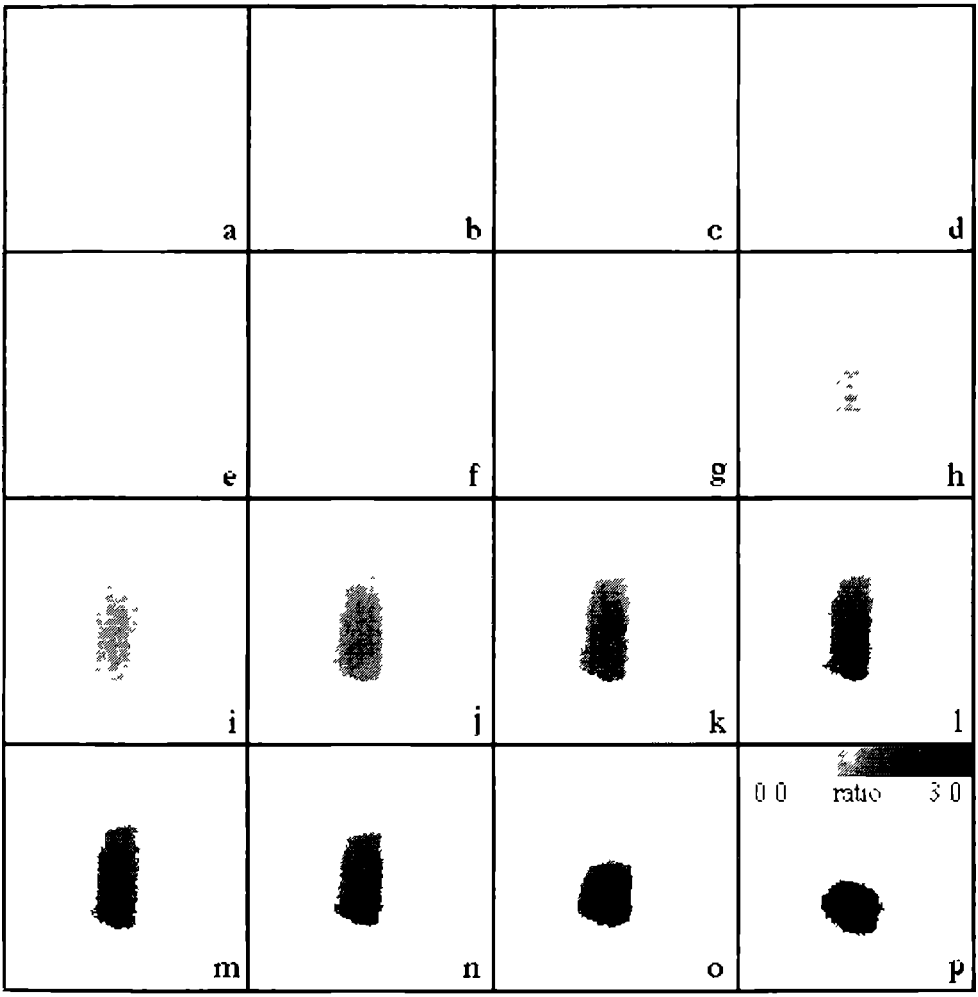


Figure 5
Digital fura-2 ratio images of an isolated rat ventricular cardiomyocyte incubated in an anoxic chamber at 37 °C. Initially, the cardiomyocyte exhibits a rod-shaped appearance with a low fura-2 ratio (images a-b). After 15 min of anoxia, the cardiomyocyte contracts and reaches the rigor state (images c-d). During the following period, the fura-2 ratio gradually increases to a maximal level (images e-m). Upon reperfusion, this particular cardiomyocyte develops hypercontraction (images n-p). The fura-2 ratio is presented in greyscales as indicated by the inserted greyscale table.

these cells $[Ca^{2+}]_i$ increased further above maximal anoxic levels (Fig. 5).

In addition to morphological observations, we used the total fura-2 fluorescence as a measure of changes in cell shape. Figures 1B and 2B demonstrate a sudden increase in total fura-2 fluorescence, indicative of a sudden contracture, within the first 15 min of anoxic incubation. On average, substrate-free anoxia induced a significant increase in total fluorescence of 31 ± 3% ($N = 25$; $P < 0.05$). As shown in figures 1B and 2B, no sudden change in cell shape occurred upon prolonged anoxia. Simultaneously with reperfusion, however, a sudden decrease in total fura-2 fluorescence can be observed in figure 2B. This is an example of a hypercontracting cell in which $[Ca^{2+}]_i$ further increases. Although the rigor contraction in the initial phase of anoxia occurred in all cardiomyocytes, the sudden increase in total fura-2 fluorescence is not always observed for unknown reasons.

Veratridine induced Ca^{2+} overload

Anoxia-induced Ca^{2-} overload in cardiomyocytes can be mimicked by addition of veratridine. Veratridine (30 μM) significantly increased $[Ca^{2-}]_i$ in freshly-isolated cardiomyocytes on coverslips from 37 ± 2 nM in nominally Ca^{2+}-free solutions (extracellular $[Ca^{2+}]$ ≈20 μM) to 787 ± 39 nM after addition of 30 μM veratridine and 2 mM $CaCl_2$ (Table I). In the presence of R 56865 (10 μM), the veratridine-induced increase in $[Ca^{2-}]_i$ was significantly reduced to 152 ± 38 nM (Table I). Veratridine (30 μM) treatment results in hypercontraction of all cardiomyocytes.

DISCUSSION

In the present study, we demonstrated that anoxia induced increases in $[Ca^{2+}]_i$ in single isolated cardiomyocytes, which either decreased or further increased upon reperfusion. In addition, the purported Na^+ overload blocker R 56865 significantly reduced anoxia-induced increases in $[Ca^{2+}]_i$.

After the isolation procedure, Ca^{2+} tolerant cardiomyocytes were obtained. Although $[Ca^{2+}]_i$ decreased from 52 ± 3 to 37 ± 2 nM at transferring the cardiomyocytes from normal Ca^{2+} (=1.25 mM) to low Ca^{2+} (≈20 μM) containing medium, no change in cell viability occurred. Similarly Cheung et al.

(1982) reported a decrease in cellular Ca^{2+} from 12.3 ± 1.8 to 3.9 ± 1.0 µmol / g protein without any change in cell viability.

During substrate-free anoxia, the isolated cardiomyocytes changed from a relaxed-elongated configuration to a contracted-square configuration which is the so-called rigor state. This change in morphology has previously been described to occur in anoxia (Hano et al., 1991; Miyata et al., 1992), hypoxia (Hohl et al., 1982) and metabolic inhibition, so-called chemical anoxia (Hohl et al., 1982; Li et al., 1989). In addition, we demonstrated that the conversion from the normal configuration to the rigor state was accompanied by an increase in total fura-2 fluorescence of 31%. The rigor state is induced by ATP depletion reached after exhaustion of endogenous substrates for the glycolytic pathway (Li et al., 1989; Hano et al., 1991). We could clearly dissociate the change in morphology and the onset of the rise in $[Ca^{2+}]_i$, since $[Ca^{2+}]_i$ was still at the normoxic level when the rigor state was reached. Individual cardiomyocytes do not reach the rigor state at the same time suggesting that ATP is depleted at different rates as each cell exhausts its glycogen stores (Li et al., 1989). After reaching the rigor contracture, $[Ca^{2+}]_i$ increased to levels well above 1 µM. Similar increases in $[Ca^{2+}]_i$ have been reported by Haigney et al. (1992). In contrast to our observations, Eisner et al. (1989) reported that $[Ca^{2+}]_i$ starts to increase before the cell contracted in experiments in which metabolic inhibition was used.

Upon reperfusion, elevated $[Ca^{2+}]_i$ either partially decreased or further increased. Whenever $[Ca^{2+}]_i$ increased above anoxic levels during reperfusion, this was accompanied by hypercontraction suggesting that elevated $[Ca^{2+}]_i$ and hypercontraction are related. In addition, cardiomyocytes developed no hypercontraction when $[Ca^{2+}]_i$ declined or remained at the anoxic level upon reperfusion. Hypercontraction and increases in $[Ca^{2+}]_i$ have also been described by Haigney et al. (1992). In contrast, others showed hypercontraction which was accompanied by a declining $[Ca^{2+}]_i$ but with elevated mitochondrial Ca^{2+} levels (Miyata et al., 1992). Next to increases in $[Ca^{2+}]_i$, we observed loss of fura-2 and decreased trypan blue exclusion in hypercontracted cardiomyocytes, which is indicative of loss of plasma membrane integrity. In this respect, it has been reported that the duration of anoxia subsequently to the appearance of the rigor state affects the probability of cardiomyocytes surviving on reperfusion (Hano et al., 1991). Since the period of anoxia after reaching the rigor state was relatively

short in the present study, only a few cardiomyocytes exhibited hypercontraction and cell death upon reperfusion.

Previously, we also measured anoxia-induced increases in $[Ca^{2+}]_i$ in renal proximal tubule cells (Table II; Rose et al., 1993; 1994). However, compared with the anoxic $[Ca^{2+}]_i$ in cardiomyocytes (> 1 μM), the anoxic $[Ca^{2+}]_i$ in proximal tubule cells was rather modest (~450 nM). Upon reperfusion the anoxic $[Ca^{2+}]_i$ recovered completely to pre-anoxic levels in the proximal tubule, whereas $[Ca^{2+}]_i$ remained high or increased in the cardiomyocytes. In addition to differences in $[Ca^{2+}]_i$, changes in cell viability were also dissimilar. In proximal tubule cells loss of cell viability occurred only during anoxia and never upon reperfusion (Rose et al., 1994). In cardiomyocytes, however, reperfusion induced additional cell death. Comparing proximal tubular cells and cardiomyocytes, we conclude that reperfusion-induced injury plays a major role in cardiomyocytes, but is absent in renal proximal tubule cells.

Table II. Effect of anoxia on $[Ca^{2+}]_i$ in isolated cardiomyocytes and renal proximal tubules

	proximal tubule cells (nM)	cardiomyocytes (nM)
normoxic $[Ca^{2+}]_i$	109 ± 2	52 ± 3#
anoxic $[Ca^{2+}]_i$	422 ± 14*	2115 ± 59*#
reperfusion $[Ca^{2+}]_i$	98 ± 3	752 ± 46*#

Values represent mean ± SE with N ≥ 38 (*P < 0.05: anoxic and reperfusion $[Ca^{2+}]_i$ versus normoxic $[Ca^{2+}]_i$; #P < 0.05: cardiomyocytes versus renal proximal tubule cells). Data for proximal tubule cells are taken from Rose et al. (1994).

Three mechanisms have been proposed to induce Ca^{2+} loading during ATP depletion and/or reperfusion.

One mechanism could be Ca^{2+} overload due to Ca^{2+} influx through voltage-gated channels, but Haworth et al. (1987) showed that Ca^{2+} channel blockers only slightly reduced increases in $[Ca^{2+}]_i$ during ATP depletion. In addition, in renal proximal tubular cells, the Ca^{2+} channel blockers methoxy-verapamil reduced anoxia-induced increases in $[Ca^{2+}]_i$ almost completely, but only slighty protected against cell death (Rose et al., 1994).

Another mechanism is ATP depletion-induced Ca^{2+} release from intracellular stores, such as the sarcoplasmic reticulum, but studies with

thapsigargine (Miyata et al., 1992) or caffeine (Allshire et al., 1987) demonstrated that the sarcoplasmic reticulum does not play a role in ATP depletion-induced Ca^{2+} overload.

The most likely mechanism is that Ca^{2+} enters the cell in exchange for accumulated Na^+ via reversed operation of the Na^+-Ca^{2+} exchanger. In this respect, it has been reported that removal of extracellular Na^+ abolished increases in $[Ca^{2+}]_i$ (Haigney et al., 1992) and that Na^+ accumulates via voltage-gated Na^+ channels during the rigor state (Tani et al., 1989; Haigney et al., 1992). The results of the present study also suggest involvement of high $[Na^+]_i$ in inducing increases in $[Ca^{2+}]_i$ during anoxia, since anoxic incubation in the presence of the purported Na^+ overload blocker R 56865 reduced significantly anoxic elevated $[Ca^{2+}]_i$. Haigney et al. (1992) showed that R 56865 markedly blunted increases in $[Na^+]_i$. It is highly unlikely that R 56865 reduces elevated $[Ca^{2+}]_i$ by blocking Ca^{2+} influx via L-type channels, since R 56865 has Ca^{2+} channel blocking properties only at concentrations exceeding 10 μM (Hano et al., 1991). In our study, we used 1 μM, well below this concentration. In contrast to cardiomyocytes, the Na^+-Ca^{2+} exchanger is absent in renal proximal tubule cells (Yu et al., 1992; Reilly et al., 1993). Therefore, anoxia-induced increases in $[Ca^{2+}]_i$ are not mediated by the Na^+-Ca^{2+} exchanger in proximal tubule cells. The most likely Ca^{2+} entry pathway in these cells are L-type Ca^{2+} channels since the Ca^{2+} channel blocker methoxyverapamil abolished elevated $[Ca^{2+}]_i$ during anoxia.

In view of the fact that anoxia-induced increases in $[Ca^{2+}]_i$ in cardiomyocytes were only partly reduced by R 56865, other pathways besides Na^+ channels must be involved in Na^+ influx during anoxia. It has been reported that in ischemic rat heart the Na^+-H^+ exchanger contributes to the cellular gain of Na^+ (Tani et al., 1989). The Na^+-H^+ exchanger has also been shown to be involved in reperfusion-induced injury (Karmazyn et al., 1993; Ver Donck et al., 1993). During ATP depletion, Na^+ efflux via (Na^+-K^+)-ATPase does not longer occur and any aspecific non-mediated leak will contribute to an increase in $[Na^+]_i$. R 56865 has also been shown to block Na^+-dependent K^+ channels in cardiomyocytes (Luk et al., 1990). Simultaneously operating K^+ efflux and Na^+ influx could in theory accelerate Na^+ overload. Therefore, blocking K^+ efflux by R 56865 may significantly delay Na^+ overload in cardiomyocytes.

We showed that anoxia-induced increases in $[Ca^{2+}]_i$ can be mimicked, albeit less profoundly, by veratridine, a compound which has been shown to open voltage-dependent Na^+ channels in excitable tissues (Pauwels et al., 1989). The veratridine experiments confirm the involvement of cytosolic Na^+ in anoxia-induced Ca^{2+} overload. The purported Na^+ overload blocker R 56865 not only reduced $[Ca^{2+}]_i$ during anoxia, but also during incubation with veratridine. Moreover, others have reported protective effects of R 56865 in the veratridine-induced cell injury (Ver Donck et al., 1991). Despite the fact that anoxia and veratridine induce increases in $[Ca^{2+}]_i$, both models are distinctively different. For example, $[Ca^{2+}]_i$ is much higher during anoxia (» 1 µM) than after incubation with veratridine (787 ± 39 nM). In addition, veratridine-induced increases in $[Ca^{2+}]_i$ were always accompanied by hypercontraction of the cardiomyocytes, while the hypercontractive state was never reached during anoxia despite the fact that $[Ca^{2+}]_i$ was much higher. This can be explained by the fact that activation of myofilaments is involved in hypercontraction, a process which needs ATP and elevated $[Ca^{2+}]_i$. During anoxia, however, ATP depletion occurs, which prevents the development of hypercontraction. In this respect, Bowers et al. (1993) reported that resynthesis of ATP and hypercontraction are closely related during removal of metabolic inhibitors. It is obvious that the combination of hypercontraction and high $[Ca^{2+}]_i$ after incubation with veratridine, is more detrimental to cardiomyocytes than high $[Ca^{2+}]_i$ alone which is obtained during anoxia. Therefore, protective effects of potential anti-ischemic drugs against cell injury induced by veratridine, should be extrapolated with caution to the ischemic myocard. An anoxic model, as used in the present study, is in our view a more suitable model to investigate protective effects of these compounds.

CHAPTER 8

General Discussion

The study described in this thesis shows that anoxia induces increases in $[Ca^{2+}]_i$ in renal PT, mTAL and cTAL cells. Due to the glycolytic capacity of cultured renal epithelial cells, however, increases in $[Ca^{2+}]_i$ were only apparent in the absence of glucose and acetate (chapter 2). Therefore, glucose and acetate were excluded from the medium to prevent ATP production via glycolysis. The anoxia-induced increases in $[Ca^{2+}]_i$ measured in PT, mTAL and cTAL cells were heterogeneously in onset and in the maximal level reached. The average maximal anoxic $[Ca^{2+}]_i$, however, was between 400 and 500 nM in PT, mTAL and cTAL cells. In view of the $Ca^{2+}{}_o$ concentration of 2 mM, this anoxic $[Ca^{2+}]_i$ value is rather modest. The stabilization of the anoxic $[Ca^{2+}]_i$ at such a low level, suggests the presence of some kind of endogenous autoprotective mechanism whereby the ATP-depleted state decreases the Ca^{2+} permeability of the plasma membrane preventing the cells from being flooded with Ca^{2+}. These observations are in line with previous studies with cultured PT cells where metabolic inhibition also induced rather small increases in $[Ca^{2+}]_i$ (McCoy et al., 1988; Phelps et al., 1989; Smith et al., 1992).

Since anoxia-induced increases in $[Ca^{2+}]_i$ in cultured (chapter 2) and freshly-isolated (chapter 3) PT cells were completely suppressed by removing $Ca^{2+}{}_o$, intracellular stores most likely do not contribute to Ca^{2+} overload during anoxia. Similar results have been reported by Smith et al. (1992) for cultured PT cells. In contrast, however, in the loop of Henle increases in $[Ca^{2+}]_i$ were only partly reduced by preventing Ca^{2+} influx, indicating that intracellular stores contribute to anoxia-induced increases in $[Ca^{2+}]_i$ in addition to Ca^{2+} influx. Moreover, when mTAL and cTAL cells are compared it is apparent that the Ca^{2+} influx component was more pronounced in mTAL than in cTAL cells (chapter 6). Besides the different sources for Ca^{2+} inducing increases in $[Ca^{2+}]_i$ during anoxia, no substantial differences in susceptibility for anoxia-induced cell injury were observed between cultured PT, mTAL or cTAL cells. Therefore, it can be concluded that differences found *in vivo* (Brezis, 1993) most likely resulted from hemodynamic factors instead of differences in intrinsic properties of the various cell types along the nephron. Nevertheless, one can not exclude the possibility that differences in intrinsic properties have been lost during culturing. This, however, seems not very likely since the culturing period was as short as possible.

Disruption of intracellular Ca^{2+} homeostasis may be an important mediator in the development of cell injury during ischemia, hypoxia or anoxia

(Nicotera et al., 1990; Farber, 1991). Increased $[Ca^{2+}]_i$ can be involved in a number of processes such as perturbation of cytoskeletal organization and activation of degradative enzymes (Brezis, 1993). As a result, increases in $[Ca^{2+}]_i$ induce cell injury which has been shown in PT cells (Phelps et al., 1989), hepatocytes (Sakaida et al., 1991) and cardiomyocytes (Buja et al., 1988; Tani et al., 1989, 1990; Haigney et al., 1992; Jeremy et al., 1992). If Ca^{2+} is a mediator of cell injury resulting from oxygen deprivation, than prevention of increased $[Ca^{2+}]_i$ should prevent cell death. In this respect, anoxic incubation of PTs in low Ca^{2+} medium reduced cell death (Takano et al., 1985; Wetzels et al., 1993). In addition, lowering $[Ca^{2+}]_o$ prevented the development of hypoxic TAL necrosis in the isolated perfused kidney (Shanley et al., 1991). Moreover, L-type Ca^{2+} channel blockers have been proven to be protective when present during an ischemic insult (Almeida et al., 1992; Burke et al., 1992; Wetzels et al., 1993), which also points to a mediating role for Ca^{2+}. In the present study, however, cell death did not occur after 60 min of anoxia in cultured PT, mTAL or cTAL cells, in spite of the elevated $[Ca^{2+}]_i$. This indicates that elevated $[Ca^{2+}]_i$ and cell death are not strictly coupled in these cells. Nevertheless, the Ca^{2+} channel blocker methoxyverapamil reduced anoxia-induced increases in $[Ca^{2+}]_i$, which identifies L-type Ca^{2+} channels as anoxia-mediated Ca^{2+} entry pathways. Evidence for such channels in the PT originate from studies on cell volume regulation (McCarty et al., 1991a, 1991b). In addition, the presence of L-type Ca^{2+} channels in TAL has been demonstrated in mixed cultures of TAL and convoluted tubules in which PTH-induced increases in $[Ca^{2+}]_i$ could be significantly inhibited by Ca^{2+} channel blockers.

In contrast to the cultured epithelial cells, anoxia-induced increases in $[Ca^{2+}]_i$ were accompanied by loss of cell viability within 1 h of anoxia in freshly-isolated PT cells (chapter 3). This clearly demonstrates that freshly-isolated PT cells are more sensitive to anoxic injury than cultured PT cells. Previously, different tolerances for hypoxia have also been described for hepatoma cells and hepatocytes (Hugo-Wisseman et al., 1991). When the anoxia-induced increases in $[Ca^{2+}]_i$ in freshly-isolated PT cells were almost completely reduced by methoxyverapamil, only a slight increase in cell viability was realized. This suggests that Ca^{2+} plays no major role in inducing cell injury. In contrast, anoxic incubation in Ca^{2+}-free medium but with 0.1 mM La^{3+}, not only abolished anoxia-related increases in $[Ca^{2+}]_i$, but also markedly improved

the cell viability. Since exposure to anoxia in Ca^{2+}-free medium, with or without EGTA, could not prevent cell death although it reduced anoxic $[Ca^{2+}]_i$, the increase in cell viability is probably not the result of reducing $[Ca^{2+}]_i$. La^{3+} most likely protects against anoxia-induced cell injury via stabilization of the plasma membrane. Also glycine provided protection against anoxia-induced cell death without affecting elevated $[Ca^{2+}]_i$. This observation again proved that elevated $[Ca^{2+}]_i$ and cell death are not causally related in renal epithelial cells.

Although anoxia induced similar increases in $[Ca^{2+}]_i$ in both cultured (chapter 2) and freshly-isolated (chapter 3) PT cells, only the freshly-isolated cells were deadly injured after 1 h of anoxia, indicative of a difference in sensitivity to oxygen deprivation. In this respect, others have demonstrated that extracellular acidosis can enhance resistance to damaging effects of oxygen deprivation in renal cells (Weinberg 1985; Burnier et al., 1988; Shanley et al., 1988; Weinberg et al., 1991a; Zager et al., 1993), cardiomyocytes (Bond et al., 1991; Koop et al., 1992) and hepatocytes (Gores et al., 1988, 1989b; Nieminen et al., 1990; Harrison et al., 1991). Chapter 4 showed that anoxia was accompanied by intracellular acidification in cultured PT cells but not in freshly-isolated PT cells. Since cell death only occurred in the freshly-isolated PT cells in which no acidification occurred, low pH_i may be a protective factor in anoxia-induced cell injury in cultured PT cells. By subjecting freshly-isolated PT cells to a low pH during anoxia, it could be demonstrated that acidosis is also protective in freshly-isolated PT cells. As stated in chapter 4, several mechanisms exist by which acidosis may be protective: inhibition of phospholipid degradation (Harrison et al., 1991), preservation of energy (Hayashi et al., 1992; Koop et al., 1992) or preservation of ion gradients across the plasma membrane during anoxia (Bonventre et al., 1985; Weinberg, 1985). The most likely mechanism in the kidney, however, is the stabilization of the plasma membrane (Bell et al., 1971). In addition, since La^{3+} and acidosis reduce anoxia-induced cell death similarly, stabilization of the plasma membrane could be a common factor in protecting cells against anoxic injury.

In most chapters of this thesis (chapters 2-4 , 6 and 7) an anoxic chamber was used to investigate the effect of anoxia on cell viability and $[Ca^{2+}]_i$. However, several other models such as hypoxia or chemical anoxia can be used to mimic ATP depletion evolving from *in vivo* ischemia. Especially the chemical anoxia model, in which metabolic inhibitors induce severe ATP depletion, is

often used in renal (Weinberg et al., 1991b, 1992; Smith et al., 1992) and liver cells (Gores et al., 1988, 1989a, 1989b; Harman et al., 1990; Harrison et al., 1990; Nieminen et al., 1990; Marsh et al., 1993b). To compare the effects of ATP depletion evolving from anoxia or from metabolic inhibition, the chemical anoxia model was tested in chapter 5. In contrast to anoxia, chemical anoxia elicited by CCCP did not result in changes in either $[Ca^{2+}]_i$ or cell viability in cultured PT cells. Only incubation with the mitochondrial uncoupler CCCP in combination with the Ca^{2+} ionophore ionomycin caused increases in $[Ca^{2+}]_i$. In addition, cell viability was severely reduced. However, the loss of cell viability in the latter condition was only in part Ca^{2+}-dependent, suggesting that elevated $[Ca^{2+}]_i$ and cell death are not strictly correlated. Similar conclusions were reached in chapter 3. Nevertheless, conclusions from the chemical anoxia model should be extrapolated with care to the anoxic model. Increases in $[Ca^{2+}]_i$ induced by CCCP and ionomycin, for example, were also induced by ionomycin alone. In addition, both incubations exhibited a similar loss of cell viability which were in part Ca^{2+}-independent. These results indicate that ionomycin does not cause cell death solely by inducing increases in $[Ca^{2+}]_i$, but has also membrane damaging properties on its own. As a result, not only the effects of ATP depletion and increased $[Ca^{2+}]_i$ are studied, but also the effect of membrane damage. Moreover, the presence of oxygen during chemical anoxia could lead to additional cell injury induced by reactive oxygen metabolites (Gores et al., 1989a; Dawson et al., 1993).

A surprising observation in this thesis is that renal epithelial cells responded rather modestly to anoxic incubation with respect to $[Ca^{2+}]_i$. Since the literature reported that a much higher anoxic $[Ca^{2+}]_i$ was observed in excitable cells (Haigney et al., 1992) than the values found in the present study for renal cells, the response of cardiomyocytes to substrate-free anoxia was investigated. Cardiomyocytes exhibited anoxia-induced increases in $[Ca^{2+}]_i$ a factor 5 higher than those observed in renal epithelial cells. In addition, reperfusion resulted in some cases in an additional increase in $[Ca^{2+}]_i$ which was never seen in the epithelial cells. Moreover, the cardiomyocytes in which $[Ca^{2+}]_i$ increased above the anoxic level during reperfusion, lost viability. In contrast, freshly-isolated PT cells that survived the anoxic incubation, always survived the following reperfusion period. These results indicate that non-excitable cells are more resistant to anoxic incubation than excitable cells such as cardiomyocytes.

Moreover, Ca^{2+} overload plays an important role in anoxia-induced cell death in cardiomyocytes, whereas in renal epithelial cells real Ca^{2+} overload does not occur. Both the anoxic and the chemical anoxic model revealed that Ca^{2+} is not the most important factor in the induction of cell injury and cell death resulting from ATP depletion in renal epithelial cells. Therefore, other events have to play a more crucial role.

The plasma membrane, for instance, is an important target in ischemic insults. Since the plasma membrane is the permeability barrier between the extracellular and the intracellular compartment, disruption of this membrane has dramatic consequences for the cell integrity and thus cell viability (Humes, 1986). In this respect, loss of phospholipid mass by ischemia-activated phospholipases and membrane disrupting effects of the degradation products, may contribute to ischemia-induced cell death (Bonventre, 1993; Weinberg, 1991). In this respect, we postulated in chapter 3 that La^{3+} protected freshly-isolated PT cells during anoxia by stabilizing the plasma membrane.

Besides the plasma membrane, the cytoskeleton might be affected leading to loss of cell-cell and cell-substrate detachment (Goligorsky et al., 1993). Additionally, alterations in the cytoskeleton lead to loss of cell polarity in epithelial cells as has been indicated by detection of the basolateral membrane enzyme Na^+-K^+-ATPase in the apical membrane after an ischemic insult (Molitoris, 1991a, 1991b). Structural rearrangements of the plasma membrane and the cytoskeleton have been described especially for the proximal tubule, in which microvilli of the brush border membrane are shed into the lumen and tight junctions became more permeable during ischemia (Weinberg, 1991). We also could detect changes in the brush border membrane of cultured PT cells during anoxia by electron microscopy and by the loss of the brush border enzyme alkalic phosphatase (data not shown). In addition, degradative cytosolic proteolysis contributes to cell injury following ATP depletion (Nicotera et al., 1988; Lee et al., 1991; Dickson et al., 1992). In this respect, glycine has been proposed to protect during anoxia by inhibiting cellular proteases activated during ischemic insults in hepatocytes (Ferguson et al., 1993).

In conclusion, the present study clearly demonstrates that although the intracellular Ca^{2+} homeostasis is disrupted during anoxia, this is not the major factor in inducing cell injury or cell death. Therefore, potentially protective agents in acute renal failure resulting from an ischemic insult, should not be sought in the prevention of increases in $[Ca^{2+}]_i$. The introduction of a low intracellular pH during the insult will be much more successful than the presence of Ca^{2+} channel blockers. In addition, the intracellular pH should be kept low at least in the initial period of reperfusion. This will probably minimize injury that develops during reperfusion. Moreover, membrane stabilizing agents such as La^{3+}, or inhibitors of proteolytic enzymes, for example, glycine (Dickson et al., 1992), should also be present during the ischemic insult. The difficulty with these protective agents is, however, that they are only useful in a laboratory setting and not during an *in vivo* ischemic insult. When the ischemic insult occurs spontaneously *in vivo*, agents which induce repair of injured cells should be searched for. These agent, however, have not been developed yet. Therefore, more research has to be done to clarify the process of cell injury and to develop protective agents that are useful after an ischemic insult.

SUMMARY / SAMENVATTING

SUMMARY

During renal ischemia, cessation of renal blood flow leads to severe oxygen and metabolic substrate deprivation, and to accumulation of waste products and metabolic products. As a result renal damage occurs leading to loss of renal function. *In vivo* studies revealed an accumulation of Ca^{2+} in the ischemic kidney, which implies an important role for Ca^{2+} in generating cell damage. Moreover, Ca^{2+} channel blockers were proven to act protective whenever present during the ischemic insult. However, it remained unclear whether accumulated Ca^{2+} was responsible for the ischemic cell injury, or was the result of cell injury and cell death. In the present study, the role of Ca^{2+} during ischemia-induced cell injury was further investigated. Therefore, an *in vitro* anoxic model was developed in which $[Ca^{2+}]_i$ could be measured during anoxic incubation of cultured and freshly-isolated renal epithelial cells using fluorescent probes.

In chapter 1 the general introduction provides a review of the current literature and the aim of the performed studies. Cellular processes such as the disturbance of the intracellular ion homeostasis, disruption of the cytoskeleton and ATP depletion, are described in detail. In addition, the experimental approaches that were used in investigating ischemia-induced cell injury are shortly described.

In chapter 2 it is shown that substrate-free anoxia induced increases in $[Ca^{2+}]_i$ in cultured proximal tubule (PT) cells, resulting from Ca^{2+} influx. The Ca^{2+} influx could be partly reduced by the L-type Ca^{2+} channel blocker methoxyverapamil (D600). Despite of increases in $[Ca^{2+}]_i$ during anoxia, however, no change in cell viability was observed, suggesting that no correlation exists between increases in $[Ca^{2+}]_i$ and loss of cell viability.

In chapter 3 it is reported that in freshly-isolated PT cells, substrate-free anoxia induced increases in $[Ca^{2+}]_i$ which are accompanied by loss of cell viability. The Ca^{2+} channel blocker D600 significantly reduced the increases in $[Ca^{2+}]_i$, suggesting that Ca^{2+} influx causes anoxic increases in $[Ca^{2+}]_i$. In addition, removal of Ca^{2+}_o completely abolished anoxia-induced increases in $[Ca^{2+}]_i$, confirming that elevated $[Ca^{2+}]_i$ results from Ca^{2+} influx. Despite the reduction of anoxic $[Ca^{2+}]_i$ by D600, however, cell viability was only slightly

improved, indicating that elevated $[Ca^{2+}]_i$ and cell death are not tightly coupled. In addition, glycine improved cell viability after 1 h of anoxia, without affecting the anoxic $[Ca^{2+}]_i$. From these results we concluded that cell injury which is unrelated to $[Ca^{2+}]_i$ is a more prominent factor in anoxia-induced cell injury in rabbit PT cells than $[Ca^{2+}]_i$ related injury.

The studies described in chapter 4 revealed that anoxia is accompanied by a decline in pH_i in cultured PT cells, but not in freshly-isolated cells. Since cultured PT cells exhibited no loss of cell viability after 1 h of anoxia, whereas cell viability decreased with approximately 50% in freshly-isolated PT cells, this cellular acidosis seems to be a protective mechanism against anoxia-induced cell injury. Reducing the pH_i during anoxia to 6.6 and 6.1 in freshly-isolated PT cells, improved cell viability, which confirms that cellular acidification has a protective effect. This chapter also revealed that elevated $[Ca^{2+}]_i$ and cell death are not directly correlated, since acidosis only slightly reduced anoxic $[Ca^{2+}]_i$ whereas cell viability increased substantially.

In chapter 5, the effect of chemical anoxia on cell viability of cultured PT cells was studied. Incubation of PT cells with the mitochondrial uncoupler CCCP in glucose-free medium, slightly decreased cell viability, but had no effect on $[Ca^{2+}]_i$. Only after the addition of the Ca^{2+} ionophore ionomycin, did $[Ca^{2+}]_i$ increase and cell viability decrease dramatically. However, although the $[Ca^{2+}]_i$ reached with CCCP plus ionomycin was substantially higher (> 1 μM) than the level reached during anoxia (~450 nM), this could not explain the fact that cell viability decreased more with CCCP and ionomycin than during anoxia. Preventing increases in $[Ca^{2+}]_i$ by incubating PT cells with CCCP and ionomycin in Ca^{2+}-free medium resulted only in a slight increase in cell viability. This result implies that the Ca^{2+} ionophore ionomycin has toxic effects on its own, which are not related to its effect on $[Ca^{2+}]_i$.

In vivo ischemia induces cell injury which is heterogeneously distributed along the nephron, indicating that the tubule segments along the nephron have different susceptibilities for ischemic cell injury. In chapter 6, the effect of substrate-free anoxia was studied in cultured mTAL and cTAL cells to investigate whether intrinsic properties of these cell types result in different susceptibilities to anoxia-induced injury. Anoxia induced increases in $[Ca^{2+}]_i$ in both mTAL and cTAL cells without a significant loss in cell viability. The anoxic $[Ca^{2+}]_i$ reached was comparable to the level reached in cultured PT cells

(~450 nM). However, elevated $[Ca^{2+}]_i$ was not only caused by Ca^{2+} influx, as is the case in PT cells, but also by Ca^{2+} release from intracellular stores. The involvement of the intracellular stores in anoxia-induced increases in $[Ca^{2+}]_i$ was less profound in mTAL than in cTAL cells. In addition, D600 reduced anoxic $[Ca^{2+}]_i$ in cTAL more than in mTAL cells.

Since anoxia-induced increases in $[Ca^{2+}]_i$ were rather modest in renal epithelial cells, anoxic experiments were also performed with cardiomyocytes in chapter 7. Anoxia induced increases in $[Ca^{2+}]_i$ well above the level reached in renal epithelial cells: ~450 nM and >1 μM in renal epithelial cells and cardiomyocytes, respectively. In addition, reperfusion occasionally induced increases in $[Ca^{2+}]_i$ above the anoxic level in the cardiomyocytes, which was never observed in renal epithelial cells. The reperfusion-induced increases in $[Ca^{2+}]_i$ were accompanied by hypercontraction and resulted in cell death. Both anoxic and reperfusion $[Ca^{2+}]_i$ could be reduced by the purported Na^+ overload blocker R 56865.

Finally, in chapter 8 the previous chapters are evaluated leading to the general conclusion that disturbance of the intracellular Ca^{2+} homeostasis is not the most prominent factor in anoxia-induced cell death in renal epithelial cells. These cells survive increases in $[Ca^{2+}]_i$ as long as the plasma membrane is intact. It is concluded that stabilization of the plasma membrane, acidosis and glycine offer protective mechanisms against anoxia-induced cell injury.

SAMENVATTING

Renale ischemie is het proces waarbij een blokkade van de bloedtoevoer leidt tot een tekort aan zuurstof en substraat, en tot een opeenhoping van afvalprodukten. Ten gevolge hiervan treedt schade aan het nierweefsel op, hetgeen uiteindelijk tot het verlies van nierfunktie leidt. Studies die een accumulatie van Ca^{2+} aantonen in de door ischemie beschadigde nier, impliceren een belangrijke rol voor Ca^{2+} in het ontstaan van celschade en celdood. Bovendien is gebleken dat Ca^{2+} kanaal blokkers een beschermend effect hebben indien ze tijdens ischemie aanwezig zijn. Het is echter nog niet duidelijk of de Ca^{2+} accumulatie in de ischemische nier de door ischemie geïnduceerde schade veroorzaakt, of juist het gevolg is van celschade. In dit proefschrift is de rol van Ca^{2+} tijdens door ischemie veroorzaakte celschade nader bestudeerd. Hiervoor is een *in vitro* anoxie model ontwikkeld waarbij met behulp van fluorescerende verbindingen de $[Ca^{2+}]_i$ gemeten kon worden tijdens anoxische incubatie van gekweekte en vers-geïsoleerde nierepitheel cellen.

In hoofdstuk 1 wordt een algemene inleiding gegeven op de achtergrond van de uit te voeren studies. Hierbij worden cellulaire processen die een rol kunnen spelen bij het ontstaan van nierschade ten gevolge van ischemie beschreven. Enkele van deze processen zijn de verstoring van de intracellulaire ion homeostase, het verlies van de cytoskeletstructuur en energieuitputting. Naast deze processen die tot schade kunnen leiden, worden er ook de experimentele modellen beschreven die gebruikt worden voor de bestudering van de door ischemie geïnduceerde schade.

In hoofdstuk 2 wordt aangetoond dat substraat-vrije anoxie stijgingen in $[Ca^{2+}]_i$ veroorzaakt in gekweekte proximale tubulus (PT) cellen. Gebleken is dat deze stijgingen het gevolg zijn van de influx van extracellulair Ca^{2+}. Deze influx van Ca^{2+} kon ten dele met de L-type Ca^{2+} kanaalblokker methoxyverapamil (D600) voorkomen worden. Ondanks de stijgingen in $[Ca^{2+}]_i$ werden er echter geen veranderingen in de celviabiliteit waargenomen, hetgeen suggereert dat stijgingen in $[Ca^{2+}]_i$ en celdood niet gecorreleerd zijn in dit experimentele model.

In hoofdstuk 3 zijn substraat-vrije anoxie experimenten uitgevoerd met vers-geïsoleerde PT cellen. Na 1 uur anoxie bleek ongeveer 50% van de verse

cellen niet meer viabel te zijn Daarnaast induceerde anoxie stijgingen in $[Ca^{2+}]_i$, die gereduceerd konden worden met de Ca^{2} kanaal blokker methoxyverapamil Dit suggereert dat de door anoxie geïnduceerde $[Ca^{2+}]_i$ stijgingen veroorzaakt worden door Ca^{2+} influx Bovendien konden anoxie geïnduceerde $[Ca^{2+}]_i$ stijgingen volledig geblokkeerd worden door Ca^{2} $_{o}$ te verwijderen De reductie van $[Ca^{2}]_i$ tijdens anoxie door D600, bood echter slechts in geringe mate bescherming tegen het optreden van celdood Dit geeft aan dat een verhoogd Ca^{2+} niveau en celdood niet direct gerelateerd zijn Daarnaast werd er bescherming door het aminozuur glycine aangetoond zonder dat stijgingen in $[Ca^{2}]_i$ werden voorkomen Uit deze resultaten werd geconcludeerd dat $[Ca^{2+}]_i$-onafhankelijke celschade een belangrijkere factor is in door anoxie geïnduceerde celschade in konijne PT cellen, dan de aan $[Ca^{2+}]_i$ gerelateerde schade

In hoofdstuk 4 is aangetoond dat de pH_i van gekweekte PT cellen sterk daalt tijdens anoxie, en dat dit niet het geval is bij vers-geïsoleerde cellen Acidose bleek een beschermingsmechanisme te zijn tegen anoxie geïnduceerde celschade en celdood In gekweekte PT cellen ging anoxie namelijk gepaard met een cytosolische verzuring en bleef het optreden van celdood uit In vers-geïsoleerde PT cellen daarentegen, trad geen verzuring op, maar wel celschade Door nu de pH_i in vers-geïsoleerde PT cellen tijdens anoxie kunstmatig te verlagen tot pH 6 6 en 6 1 kon de celviabiliteit sterk verhoogd worden Overeenkomstig hoofdstuk 3 bleek er ook nu geen verband te bestaan tussen Ca^{2+} en celdood $[Ca^{2}]_i$ metingen tijdens anoxie met een kunstmatig verlaagde pH_i wezen uit dat de door anoxie geïnduceerde $[Ca^{2}]_i$ stijgingen slechts minimaal gereduceerd werden door deze lage pH_i, terwijl de celviabiliteit sterk toenam.

Ter vergelijking met het *in vitro* anoxie model is het effect van chemische anoxie op de celviabiliteit van gekweekte PT cellen bestudeerd in hoofdstuk 5 In dit model zijn monolagen van gekweekte PT cellen geïncubeerd in substraatvrij medium met de ontkoppelaar CCCP Gekweekte PT cellen vertoonden geen meetbare celschade noch stijgingen in $[Ca^{2+}]_i$ na 2 uur chemische anoxie. Pas na toediening van de Ca^{2+} ionofoor ionomycine tijdens chemische anoxie vond er een grote toename in celschade plaats De op deze wijze ontstane celdood ging gepaard met $[Ca^{2}]_i$ stijgingen boven 1 µM Dit Ca^{2+} niveau is veel hoger dan de concentratie die tijdens anoxie bereikt wordt De door chemische anoxie veroorzaakte celdood was echter maar ten dele afhankelijk van Ca^{2+} Incubaties in Ca^{2+}-vrije media, met of zonder EGTA, konden namelijk de celviabiliteit

maar in beperkte mate herstellen.

Aangezien *in vivo* studies uitwezen dat de reaktie op ischemie niet gelijk is voor de verschillende celtypen in het nefron, zijn in hoofdstuk 6 de effecten van anoxie op andere nierepitheel celtypen dan de PT cellen getest, namelijk cellen uit het corticale en medullaire stijgende deel van de lus van Henle (cTAL en mTAL cellen). In beide celtypen veroorzaakte anoxie stijgingen in $[Ca^{2+}]_i$. Het bereikte Ca^{2+} niveau was vergelijkbaar met het niveau dat in PT cellen bereikt wordt, namelijk ~450 nM. Overeenkomstig met gekweekte PT cellen, veroorzaakte 1 uur anoxie ook nu geen celdood. De stijgingen in $[Ca^{2+}]_i$ in cTAL cellen bleken veroorzaakt door zowel Ca^{2+} influx uit het medium als door Ca^{2+} efflux uit de intracellulaire stores. In mTAL cellen daarentegen was de betrokkenheid van de intracellulaire stores veel minder groot. Dit werd bevestigd door het feit dat de Ca^{2+} kanaal blokker methoxyverapamil de $[Ca^{2+}]_i$ stijgingen in mTAL cellen meer reduceert dan in cTAL cellen.

Aangezien anoxie slechts geringe stijgingen in $[Ca^{2+}]_i$ veroorzaakte in niercellen, zijn in hoofdstuk 7 anoxie experimenten uitgevoerd met ventriculaire cardiomyocyten. In cardiomyocyten werden anoxie geïnduceerde $[Ca^{2+}]_i$ stijgingen aangetoond die een factor 5 hoger liggen dan de waarden gevonden in nierepitheel cellen. Daarnaast veroorzaakte reperfusie, in tegenstelling tot niercellen, soms een verdere stijging van $[Ca^{2+}]_i$. Deze zogenaamde "Ca^{2+} paradox" ging gepaard met hypercontractie. Zowel de $[Ca^{2+}]_i$ waarden gemeten tijdens anoxie als tijdens reperfusie konden met de Na^+ entry blocker R 56865 gereduceerd worden.

Samenvattend is uit dit proefschrift gebleken dat verstoring van de intracellulaire Ca^{2+} homeostase door anoxie niet de belangrijkste oorzaak is van celdood. Nierepitheel cellen blijken in staat te zijn de verstoring van de Ca^{2+} balans te overleven mits de plasmamembraan intact is gebleven. Stabilisering van de plasmamembraan tijdens de anoxische periode lijkt een belangrijke bescherming op te leveren tijdens anoxie. Daarnaast werken acidose en glycine tijdens anoxie beschermend.

REFERENCES

Allshire A, Piper HM, Cuthbertson KSR and PH Cobbold (1987) Cytosolic free Ca^{2+} in single rat heart cells during anoxia and reoxygenation. Biochem J 244: 381-385

Almeida ARP, Bunnachak D, Burnier M, Wetzels JFM, Burke TJ and RW Schrier (1992) Time-dependent protective effects of calcium channel blockers on anoxia- and hypoxia-induced proximal tubule injury J Pharmacol Exp Ther 260 526-532

Altschuld RA, Hostetler JR and GP Brierley (1981) Response of isolated heart cells to hypoxia, reoxygenation and acidosis. Circ Res 49 307-316

Backsai BJ and PA Friedman (1990) Activation of latent Ca^{2+} channels in renal epithelial cells by parathyroid hormone. Nature 347 388-391

Bagnasco S, Good D, Balaban R and B Burg (1985) Lactate production in isolated segments of the rat nephron. Am J Physiol 248: F522-F526

Barrett AJ (1980) The many forms and functions of cellular proteinases. Fed Proc 39 9-14

Beck F, Thurau K and G Gstraunthaler (1992) Pathophysiology and Pathobiochemistry of acute renal failure, in The Kidney: Physiology and Pathology, edited by Seldin DW and G Giebisch, Raven Press, Ltd., New York, USA, pp 3157-3179

Bell ML, Lazarus HM, Herman AH, Egdahl RH and AM Rutenburg (1971) pH-dependent changes in cell membrane stability. Proc Soc Exp Biol Med 136: 298-300

Bellomo G, Fulceri R, Albano E, Gam berucci A, Pompella A, Parola M and A Benetti (1991) Ca^{2+}-dependent and independent mitochondrial damage in hepatocellular injury Cell Calcium 12: 335 341

Bershadsky AD, Gelfand VI, Svitkina TM and IS Tint (1980) Destruction of microfilament bundles in mouse embryo fibroblasts treated with inhibitors or energy metabolism. Exp Cell Res 127: 421-429

Bershadsky AD and VI Gelfand (1981) ATP-dependent regulation of cytoplasmic microtubule disassembly. Proc Natl Acad Sci USA 78: 3610-3616

Bershadsky AD and VI Gelfand (1983) Role of ATP in the regulation of stability of cytoskeletal structures. Cell Biol Int Rep 7: 173-187

Bickler PE (1992) Cerebral anoxia tolerance in turtles: regulation of intracellular calcium and pH. Am J Physiol 263: R1298-R1302

Bindels RJM, Hartog A, Timmermans J and CH Van Os (1991) Active Ca^{2+} transport in primary cultures of rabbit kidney CCD: stimulation by 1,25-dihydroxyvitamin D_3 and PTH. Am J Physiol 261: F799-F807

Bond JM, Herman B and JJ Lemasters (1991) Protection by acidotic pH against anoxia/reoxygenation injury to rat neonatal cardiac myocytes. Biochem Biophys Res Commun 179: 798-803

Bonventre JV (1984) Cellular response to ischemia, in Acute Renal Failure, edited by Solez K and A Whelton, Marcel Dekker, New York, USA, pp 195-218

Bonventre JV and JY Cheung (1985) Effects of metabolic acidosis on viability of cells exposed to anoxia. Am J Physiol 249. C149-C159

Bonventre JV (1993) Mechanisms of ischemic acute renal failure. Kidney Int 43: 1160-1178

Bowers KC, Allshire AP and PH Cobbold (1993) Continuous measurements of cytoplasmic ATP in single cardiomyocytes during simulation of the "oxygen paradox". Cardiovasc Res 27: 1836-1939

Boyarski G, Ganz MB, Sterzel RB and WF Boron (1988) pH regulation in single glomerular mesangial cells I. Acid extrusion in absence and presence of HCO_3 . Am J Physiol 255: C844-C856

Brezis M, Shanley P, Silva P, Spokes K, Lear S, Epstein FH and S Rosen (1985) Disparate mechanisms for hypoxic cell injury in different nephron segments. Studies in the isolated perfused rat kidney. J Clin Invest 76: 1796-1806

Brezis M, Rosen S and FH Epstein (1991) Acute renal failure, in The kidney, edited by Brenner BM and FC Rector, Jr., WB Saunders Company, Harcourt Brace Jovanovich, Inc., Philadelphia, USA, pp 993-1061

Brezis M (1993) Cellular mechanisms of acute ischemic injury in the kidney. Annu Rev med 44: 27-37

Bronk SF and GJ Gores (1991) Efflux of protons from acidic vesicles contributes to cytosolic acidification of hepatocytes during ATP depletion. Hepatology 14: 626-633

Brown BL, Albano JDM, Ekins RP and AM Sgherzi (1971) A single and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. Biochem J 121: 561-562

Buja LM, Hagler HK and JT Willerson (1988) Altered calcium homeostasis in the pathogenesis of myocardial ischemic and hypoxic injury. Cell Calcium 9: 205-217

Burke TJ, Arnold PA, Gordon JA, Bulger RE, Dobayn DC and RW Schrier (1984) Protective effect of intrarenal calcium membrane blockers before or after renal ischemia. J Clin Invest 74: 1830-1841

Burke TJ and RW Schrier (1992) Pathophysiology of cell ischemia, in Diseases of the Kidney, edited by Schrier RW, Gottschalk CW, Little, Brown, Boston, Toronto, London, pp 1257-1286

Burnier M, Putten van VJ, Schieppati A and RW Schrier (1988) Effect of extracellular acidosis on ^{45}Ca uptake in isolated hypoxic proximal tubules. Am J Physiol 254: C839-C846

Busa WB and R Nuccitelli (1984) Metabolic regulation via intracellular pH. Am J Physiol 246: R409-R438

Canfield PE, Geerdes AM, Molitoris BA (1991) Effect of reversible ATP depletion on tight-junction integrity in LLC-PK_1 cells. Am J Physiol 261: F1038-F1045

Cannon JR, Harvison PJ and GF Rush (1991) The effects of fructose on adenosine triphosphate depletion following mitochondrial dysfunction and lethal cell injury in isolated rat hepatocytes. Toxicol Appl Pharmacol 108: 407-416

Carafoli E (1987) Intracellular calcium homeostasis. Annu Rev Biochem 56: 395-433

Chan L, Ledingham JGG, Clarke J and BD Ross (1982) The importance of pH in acute renal failure, in Acute Renal Failure, edited by Eliahou HE, London, Libbey, pp 58-61

Cheung JY, Thompson IG and JV Bonventre (1982) Effects of extracellular calcium removal and anoxia on isolated rat myocytes. Am J Physiol 243: C184-C190

Cheung JY, Constantine JM and JV Bonventre (1986) Regulation of cytosolic free calcium concentration in cultured renal epithelial cells. Am J Physiol 251: F690-F701

Cotterill LA, Gowr JD, Fuller BJ and CJ Green (1989) Oxidative damage to kidney membranes during cold ischemia. Transplantation 48: 745-751

Dawson TL, Gores GJ, Nieminen A-L, Herman B and JJ Lemasters (1993) Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes. Am J Physiol 264: C961-C967

Dickman KG and LJ Mandel (1990) Differential effects of respiratory inhibitors on glycolysis in proximal tubules. Am J Physiol 258: F1608-F1615

Dickson RC, Bronk SF and GJ Gores (1992) Glycine cytoprotection during lethal hepatocellular injury from adenosine triphosphate depletion. Gastroenterology 102: 2098-2107

Doctor RB, Bennett V and LJ Mandel (1993) Degradation of spectrin and ankyrin in the ischemic rat kidney Am J Physiol 264. C1003-C1013

Donohoe JF, Venkatachalam MA, Bernard DB and NG Levinsky (1978) Tubular leakage and obstruction after renal ischemia· Structural-functional correlations. Kidney Int 13· 208-222

Eisner DA, Nichols CG, O'Neill SC, Smith GL and M Valdeolmillos (1989) The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. J Physiol 411: 393-418

Farber JL (1990) The role of calcium in lethal cell injury. Chem Res Toxicol 3· 503-508

Ferguson DM, Gores GJ, Bronk SF and RAF Krom (1993) An increase in cytosolic protease activity during liver preservation: inhibition by glutathione and glycine. Transplantation 55. 627-633

Goligorsky MS and KA Hruska (1988) Hormonal modulation of cytoplasmic calcium concentration in renal tubular epithelium. Miner Electrolyte Metab 14. 58-70

Goligorsky MS, Morgan MA, Lyubsky S, Gross RW, Adams DT and DR Spitz (1993) Establishment of a hydrogen peroxide resistant variant of renal tubular epithelial cells role of calcium-dependent phospholipase A_2 in cell damage. Arch Biochem Biophys 301: 119-128

Gores GJ, Nieminen A-L, Fleishman KE, Dawson TL, Herman B and JJ Lemasters (1988) Extracellular acidosis delays onset of cell death in ATP-depleted hepatocytes. Am J Physiol 255: C315-C322

Gores GJ, Flarsheim CE, Dawson TL, Nieminen AL, Herman B and JJ Lemasters (1989a) Swelling, reductive stress, and cell death during chemical hypoxia in hepatocytes Am J Physiol 257: C347-C354

Gores GJ, Nieminen A-L, Wray BE, Herman B and JJ Lemasters (1989b) Intracellular pH during "chemical hypoxia" in cultured rat hepatocytes. Protection by intracellular acidosis against the onset of cell death. J Clin Invest 83: 386-396

Grynkiewicz G, Poenie M and RY Tsien (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260· 3440 3450

Haigney MCP, Miyata H, Lakatta EG, Stern MD and HS Silverman (1992) Dependence of hypoxic cellular calcium loading on Na^+-Ca^{2} exchange. Circ Res 71. 547-557

Hano O, Silverman HS, Blank PS, Mellits ED, Baumgardner R, Lakatta EG and MD Stern (1991) Nicardipine prevents calcium loading and "oxygen paradox" in anoxic single rat myocytes by a mechanism independent of calcium channel blockade Circ Res 69· 1500-1505

Harman AW, Nieminen A-L, Lemasters JJ and B Herman (1990) Cytosolic free magnesium, ATP and blebbing during chemical hypoxia in cultured rat hepatocytes. Biochem Biophys Res Commun 170: 477-483

Harrison DC, Lemasters JJ and B Herman (1991) A pH-dependent phospholipase A_2 contributes to loss of plasma membrane integrity during chemical hypoxia in rat hepatocytes. Biochem Biophys Res Commun 174: 654-659

Haworth RA, Goknur AB, Hunter DR, Hegge JO and HA Berkoff (1987) Inhibition of calcium influx in isolated adult rat heart cells by ATP depletion. Circ Res 60: 586-594

Hayashi H, Miyata H, Noda N, Kobayashi A, Hirano M, Kawai T and N Yamazaki (1992) Intracellular Ca^{2+} concentration an pH, during metabolic inhibition. Am J Physiol 262. C628-C634

Herman B, Gores GJ, Nieminen A-L and JJ Lemasters (1988), in Integration of mitochondrial function, edited by Lemasters JJ, Hackenbrock CR, Thurman RG, and HV Westerhoff, Plenum, New York, USA, pp 379-391

Hochachka PW and TP Mommsen (1983) Protons and anaerobiosis. Science 219: 1391-1397

Hohl CM and RA Altschuld (1982) Response of isolated adult canine cardiac myocytes to prolonged hypoxia and reoxygenation. Am J Physiol 242: H1022-H1030

Hugo-Wisseman D, Anundi I, Lauchart W, Veibahn R and H De Groot (1991) Differences in glycolytic capacity and hypoxia tolerance between hepatoma cells and hepatocytes. Hepatology 13: 297-303

Humes HD (1986) Role of calcium in pathogenesis of acute renal failure. Am J Physiol 250: F579-F589

Ince C, Beekman RE and G Verschragen (1990) A micro-perfusion chamber for single-cell fluorescence measurements. J Immunol Methods 128: 227-234

Jacobs WR, Sgambati M, Gomez G, Vilaro P, Higdon M, Bell PD and LJ Mandel (1991) Role of cytosolic Ca in renal tubule damage induced by anoxia. Am J Physiol 260: C545-C554

Jeremy RW, Koretsure Y, Marban E and LC Becker (1992) Relation between glycolysis and calcium homeostasis in postischemic myocardium. Circ Res 70: 1180-1190

Joseph JK, Bunnachak D, Burke TJ and RW Schrier (1990) A novel method of inducing and assuring total anoxia during in vitro studies of O_2 deprivation injury. J Am Soc Nephrol 1: 837-840

Kaczorowski GJ, Slaughter RS, King F and ML Garcia (1989) Inhibitors of sodium-calcium exchange: identification and development of probes of transport activity. Biochim Biophys Acta 988: 287-302

Karmazyn M, Ray M and JV Haist (1993) Comparative effects of Na^+/H^+ exchange inhibitors against cardiac injury produced by ischemia/reperfusion, hypoxia/reoxygenation, and the calcium paradox. J Cardiovasc Pharmacol 21: 172-178

Katz AM (1985) Basic cellular mechanisms of action of the calcium-channel blockers. Am J Cardiol 55: 2B-9B

Kehrer JP, Jones DP, Lemasters JJ, Farber JL and H Jaeschke (1990) Contemporary issues in toxicology. Mechanisms of hypoxic cell injury. Toxicol Appl Pharmacol 106: 165-178

Kriz W (1988) A standard nomenclature for structures of the kidney. Am J Physiol 23: F1-F8

Koop A and HM Piper (1992) Protection of energy status of hypoxic cardiomyocytes by mild acidosis. J Mol Cell Cardiol 24: 55-65

Lash LH and JJ Tokarz (1990) Oxidative stress in isolated rat renal proximal and distal tubular cells. Am J Physiol 259: F338-F347

Lee JA and DG Allen (1991) Mechanisms of acute ischemic contractile failure of heart. Role of intracellular calcium. J Clin Invest 88: 361-367

Lemasters JJ, Diguiseppi J, Nieminen A-L and B Herman (1987) Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes. Nature 325: 78-81

Li Q, Hohl CM, Altschuld RA and BT Stokes (1989) Energy depletion-repletion and calcium transients in single cardiomyocytes. Am J Physiol 257: C427-C434

Luk H-N and E Carmeliet (1990) Na^+-activated K^+ current in cardiac cells; rectification, open probability, block and role digitalis toxicity. Pflügers Arch 416: 766-768

Malis CD, Cheung JY, Leaf AL and JV Bonventre (1983) Effects of verapamil in models of ischemic acute renal failure in the rat. Am J Physiol 245: F735-F742

Mandel LJ and E Murphy (1984) Regulation of cytosolic free calcium in rabbit proximal renal tubules. J Biol Chem 259: 11188-11196

Marsh JD and TS Smith (1993a) Calcium overload and ischemic myocardial injury. Circulation 83: 709-711

Marsh DC, Vreugdenhill PK, Mack VE, Belzer FO and JH Southard (1993b) Glycine protects hepatocytes from injury caused by anoxia, cold ischemia and mitochondrial inhibitors, but not injury caused by calcium ionophores or oxidative stress. Hepatology 17: 91-98

Mason J, Joeris B, Welsch J and W Kriz (1989) Vascular congestion in ischemic renal failure: The role of cell swelling. Miner Electrolyte Metab 15: 114-124

McCarty NA and RG O'Neil (1991a) Calcium-dependent control of volume regulation in renal proximal tubule cell. I. Swelling-activated Ca^{2+} entry and release. J Membr Biol 123: 149-160

McCarty NA and RG O'Neil (1991b) Calcium-dependent control of volume regulation in renal proximal tubule cell. II. Roles of dihydropyridine-sensitive and -insensitive Ca^{2+} entry pathways. J Membr Biol 123: 161-170

McCoy CE, Selvaggio AM, Alexander EA and JH Schwartz (1988) Adenosine triphosphate depletion induces a rise in cytosolic free calcium in canine renal epithelial cells. J Clin Invest 82: 1326-1332

Miyata H, Lakatta EG, Stern MD and HS Silverman (1992) Relation of mitochondrial and cytosolic free calcium to cardiac myocyte recovery after exposure to anoxia. Circ Res 71: 605-613

Molitoris BA, Hoilien CA, Dahl R, Ahnen DJ, Wilson PD and J Kim (1988) Characterization of ischemia-induced loss of epithelial polarity. J Membr Biol 106: 233-242

Molitoris BA, Dahl RH and SA Falk (1989) Ischemic-induced loss of epithelial polarity. Role of the tight junction. J Clin Invest 84: 1334-1339

Molitoris BA (1991a) New insights into cell biology of ischemic acute renal failure. J Am Soc Nephrol 1: 1263-1270

Molitoris BA (1991b) Ischemia-induced loss of epithelial polarity: potential role of the actine cytoskeleton (editorial). Am J Physiol 260: F769-F778

Morel F (1981) Sites of hormone action in the mammalian nephron. Am J Physiol 240: F159-F164

Muallem S, Zhang B-X, Loessberg PA and RA Star (1992) Simultaneous recording of cell volume changes and intracellular pH or Ca^{2+} concentration in single osteosarcoma cells UMR-106-01. J Biol Chem 267: 17658-17664

Nakao A, Allen ML, Sonnenburg WK, Smith WL (1989) Regulation of cAMP metabolism by PGE_2 in cortical and medullary thick ascending limb of Henle 's loop. Am J Physiol 256: C652-C657

Neylon CB, Hoyland J, Mason WT and RF Irvine (1990) Spatial dynamics of intracellular calcium in agonist-stimulated vascular smooth muscle cells. Am J Physiol 259: C675-C686

Nicotera P, Bellomo G and S Orrenius (1990) The role of Ca^{2+} in cell killing. Chem Res Toxicol 3: 484-494

Nieminen A-L, Gores GJ, Wray BE, Tanaka Y, Herman B and JJ Lemasters (1988) Calcium dependence of bleb formation and cell death in hepatocytes. Cell Calcium 9: 237-246

Nieminen A-L, Dawson TL, Gores GJ, Kawanishi T, Herman B and JJ Lemasters (1990) Protection by acidotic pH and fructose against lethal injury to rat hepatocytes from mitochondrial inhibitors, ionophores and oxidant chemicals. Biochem Biophys Res Commun 167: 600-606

Osswald H, Schmitz HJ and R Kemper (1977) Tissue content of adenosine, inosine and hypoxanthine in the rat kidney after ischemia and postischemic recirculation. Pflügers Arch 371: 45-49

Pauwels PJ, Assouw HP Van, Leysen JE and PAJ Janssen (1989) Ca^{2+}-mediated neuronal death in rat brain neuronal cultures by veratridine: protection by flunarizine. Mol Pharmacol 36: 525-531

Pentilla A and BF Trump (1974) Extracellular acidosis protects Ehrlich ascites tumor cells and rat renal cortex against anoxic injury. Science 185: 277-278

Phelps PC, Smith MW and BF Trump (1989) Cytosolic ionized calcium and bleb formation after acute cell injury of cultured rabbit renal tubule cells. Lab Invest 60: 630-642

Reilly RF, Shugrue CA, Lattanzi D and D Biemesderfer (1993) Immunolocalization of the Na^+/Ca^{2+} exchanger in rabbit kidney. Am J Physiol 265: F327-F332

Rodeheaver DP and RG Schnellmann (1990) Extracellular acidosis ameliorates metabolic inhibitor-induced and potentiates oxidative-induced cell death in rabbit proximal tubules (RPT) (abstract) Kidney Int 37: 493

Rose UM, Bindels RJM, Vis A, Jansen JWCM and CH Van Os (1993) The effect of L-type Ca^{2+} channel blockers on anoxia-induced increases in intracellular Ca^{2+} concentration in rabbit proximal tubule cells in primary culture. Pflugers Arch 423: 378-386

Rose UM, Bindels RJM, Jansen JWCM and CH Van Os (1994) Effects of Ca^{2+} channel blockers, low Ca^{2+} medium and glycine on cell Ca^{2+} and injury in anoxic rabbit proximal tubules. Kidney Int, in press

Rossi CS and AL Lehninger (1964) Stoichiometry of respiratory stimulation, accumulation of Ca^{2+} and phosphate, and oxidative phosphorylation in rat liver mitochondria. J Biol Chem 239: 3971-3980

Sakaida I, Thomas AP and JL Farber (1991) Increases in cytosolic calcium ion concentration can be dissociated from the killing of cultured hepatocytes by tert-butyl hydroperoxide J Biol Chem 266: 717-722

Schrier RW, Arnold PE, Van Putten VJ and TJ Burke (1987) Cellular calcium in ischemia acute renal failure: role of calcium entry blockers. Kidney Int 32: 313-321

Schwertschlag U, Schrier RW and P Wilson (1986) Beneficial effects of calcium channel blockers and calmodulin binding drugs on in vitro renal cell anoxia. J Pharmacol Exp Ther 238: 119-124

Schwerz DW, Kreisberg JI and MA Venkatachalam (1983) Characterization of rat kidney proximal tubule brush border membrane associated phosphatidylinositol phosphodiesterase. Arch Biochem Biophys 224: 555-567

Shanley PF, Brezis M, Spokes K, Silva P, Epstein FH and S Rosen (1986a) Differential responsiveness of proximal tubule segments to metabolic inhibitors in the isolated perfused rat kidney. Am J Kidney Dis 7: 76-83

Shanley PF, Rosen MD, Brezis M, Silva P, Epstein FH and S Rosen (1986b) Topography of focal proximal tubular necrosis after ischemia with reflow in the rat kidney. Am J Pathol 122: 462-468

Shanley PF, Shapiro JI, Chan L, Burke TJ and GC Johnson (1988) Acidosis and hypoxic medullary injury in the isolated perfused kidney. Kidney Int 34, 791-796

Shanley PF and GC Johnson (1991) Calcium and acidosis in renal hypoxia. Lab Invest 65: 298-305

Shapiro JI, Cheung C, Itabashi A, Chan A and RW Schrier (1985) The effect of verapamil on renal function after warm and cold ischemia in the isolated perfused rat kidney. Transplantation 40: 596-600

Shimizu T, Kawabata T and M Nakamura (1990) Protective effect of a novel calcium blocker, S-312-d, on ischemic acute renal failure in rat. J Pharmacol Exp Ther 255: 484-490

Siesjo BK (1989) Calcium and cell death. Magnesium 8: 223-237

Silverman M, Rose H and JB Puschett (1989) Modifications in proximal tubular function induced by nitrendipine in a rat model of acute ischemic renal failure. J Cardiovasc Pharmacol 14: 799-802

Smith MT, Thor H and S Orrenius (1981) Toxic injury to isolated hepatocytes is not dependent on extracellular calcium. Sciences 213: 1257-1259

Smith MW, Phelps PC and BF Trump (1992) Injury-induced changes in cytosolic Ca^{2+} in individual rabbit proximal tubule cells Am J Physiol 262: F647-F655

Snedecor GW and WG Cochran (1974) Statistical methods. The Iowa state University Press, Ames, Iowa

Snowdowne KW, Freudenrich CC and AB Borle (1985) The effects of anoxia on cytosolic free calcium, calcium fluxed, and cellular ATP levels in cultured kidney cells J Biol Chem 260.11619-11626

Steenbergen C, Murphy E, Watts JA and RE London (1990) Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat heart. Circ Res 66. 135-146

Sugrue SP and EA Hay (1981) Responses of basal epithelial cell surface and cytoskeleton to solubilized extracellular matrix molecules J Cell Biol 91: 45-54

Takano T, Soltoff SP, Murdaugh S and LJ Mandel (1985) Intracellular respiratory dysfunction and cell injury in short-term anoxia of rabbit renal proximal tubules. J Clin Invest 76 2377-2384

Talén PG, Nordlander MIL, Sohtell MEH and LET Svensson (1991) Attenuation of renal ischaemic injury by felodipine Arch Pharm 343: 411-417

Tang M-J, Suresh KR and RL Tannen (1989) Carbohydrate metabolism by primary cultures of rabbit proximal tubules. Am J Physiol 256: C532-C539

Tani M and JR Neely (1989) Role of intracellular Na^+ in Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts Possible involvement of H^+-Na^+ and Na^+-Ca^{2+} exchange Circ Res 65. 1045-1056

Tani M (1990) Mechanisms of Ca^{2+} overload in reperfused ischemic myocardium. Annu Rec Physiol 52: 543-559

Tauc M, Le Maout S and P Poujeol (1990) Fluorescent video-microscopy study of regulatory volume decrease in primary culture of rabbit proximal convoluted tubule. Biochim Biophys Acta 1052· 278-284

Thevenod F, Streb H, Ulrich KJ and I Schulz (1986) Inositol-1,4,5-triphosphate releases Ca^{2+} from a nonmitochondrial store site in permeabilized rat cortical kidney cells Kidney Int 29: 695-702

Thomas CE and DJ Reed (1988) Effect of extracellular Ca^{2+} omission on isolated hepatocytes. I. Induction of oxidative stress and cell injury. J Pharmacol Exp Ther 245: 493-500

Thomas CE and DJ Reed (1988) Effect of extracellular Ca^{2+} omission on isolated hepatocytes. II. Loss of mitochondrial membrane potential and protection by inhibition of uniport Ca^{2+} transduction J Pharmacol Exp Ther 245: 501-507

Toutain H, Vauclin-Jacques N, Fillastre J-P and J-P Morin (1991) Biochemical, functional, and morphological characterization of primary culture of rabbit proximal tubule cells Exp Cell Res 194: 9-18

Turrens JS, Alexander A and AL Lehninger (1985) Ubisemiquinone is the electron donor for suproxide formation by complex III of heart mitochondria. Arch Biochem Biophys 237: 408-414

Uchida S and H Endou (1988) Substrate specificity to maintain cellular ATP along the mouse nephron Am J Physiol 255: F977-F983

Venkatachalam MA, Bernard DB, Donohoe JF and NG Levinsky (1978) Ischemic damage and repair in the rat proximal tubule Differences among S1, S2 and S3 segments. Kidney Int 14: 31-49

Ver Donck L and M Borgers (1991) Myocardial protection by R 56865: a new principal based on prevention of ion channel pathology. Am J Physiol 261: H1828-H1835

Ver Donck L, Borgers M and F Verdonck (1993) Inhibition of sodium and calcium overload pathology in the myocardium: a new cytoprotective principle. Cardiovasc Res 27 349-357

Verity MA, Torres M and T Sarafian (1991) Paradoxical potentiation by extracellular Ca^{2+} of acute chemical anoxic neuronal injury in cerebellar granule cell culture. Mol Chem Neuropathology 15: 271-233

Wagner K, Albrecht S and NH Neumayer (1987) Prevention of posttransplant acute tubular necrosis by the calcium antagonist diltiazem: A prospective randomized study. Am J Nephrol 7: 287-291

Watts JA, Koch CD and KF La Noue (1980) effects of Ca^{2+} antagonists on energy metabolism: Ca^{2+} and heart function after ischemia. Am J Physiol 238: H909-H916

Webb DL (1966) Enzyme and metabolic inhibitors (vol. III). New York, Academic Press, pp 1-283

Welle ter HF, Baartscheer A and JWT Fiolet (1988) The cytoplasmic free energy of ATP hydrolysis in isolated rod shaped rat ventricular myocytes. J Mol and Cell Cardiology 20; 435-441

Weinberg JM (1985) Oxygen deprivation-induced injury to isolated rabbit kidney tubules. J Clin Invest 76: 1193-1208

Weinberg JM (1991) The cell biology of ischemic renal injury. Kidney Int 39: 476-500

Weinberg JM, Davis JA, Roeser NF and MA Venkatachalam (1991a) Role of increased cytosolic free calcium in the pathogenesis of rabbit proximal tubule cell injury and protection by glycine or acidosis. J Clin Invest 87: 581-590

Weinberg JM, Venkatachalam MA, Roeser NF, Davis JA, Varani J and KJ Johnson (1991b) Amino acid protection of cultured kidney tubule cells against calcium ionophore-induced lethal cell injury. Lab Invest 65: 671-678

Weinberg JM, Varani J, Johnson KJ, Roeser NF, Dame MK, Davis JA and MA Venkatachalam (1992) Protection of human umbilical vein endothelial cells by glycine and structurally similar amino acids against calcium and hydrogen peroxide-induced lethal cell injury. Am J Pathol 140: 457-471

Wetzels JFML, Yu L, Wang X, Kribben A, Burke TJ and RW Schrier (1993) Calcium modulation and cell injury in isolated rat proximal tubules. J Pharmacol Exp Ther 267: 176-180

Wilson PD and RW Schner (1986) Nephron segment and calcium as determinants of anoxic cell death in primary renal cell cultures. Kidney Int 29: 1172-1179

Yin HL and TP Stossel (1979) Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium dependent regulatory protein. Nature 281: 583-586

Young EW and HD Humes (1991) Calcium and acute renal failure. Miner Electrolyte Metab 17: 106-111

Yu ASL, Hebert SC, Lee S-L, Brenner BM and J Lytton (1992) Identification and localization of renal Na^{+}-Ca^{2+} exchanger by polymerase chain reaction. Am J Physiol 263: F680-F685

Zager RA, Schimpf BA and DJ Gmur (1993) Physiological pH. Effects on posthypoxic proximal tubular injury. Circ Res 72: 837-846

DANKWOORD

Om te voorkomen dat ik iemand vergeet, wil ik beginnen met een ieder te bedanken die bijgedragen heeft aan het tot stand komen van dit proefschrift. Er zijn echter een aantal mensen tot wie ik me persoonlijk wil richten.

Allereerst natuurlijk mijn promotor prof. dr. C.H. van Os: Carel, gedurende de afgelopen jaren heb ik zeer veel van je mogen leren. Hiervoor wil ik je van harte bedanken. Daarnaast dank ik je ook voor je vertrouwen, steun en enthousiasme. De vele uren die wij samen in dit proefschrift gestoken hebben, hebben zich geloond. Dat je niet alleen een goede "prof" bent, maar ook een prettige reisgenoot is wel gebleken tijdens ons congresbezoek in New Orleans in de VS. Ook daarvoor dank ik je.

Daarnaast wil ik mijn copromotoren dr. R.J.M. Bindels en dr. J.W.C.M. Jansen bedanken. René, jij niet alleen bedankt voor alle steun en hulp, maar ook voor je ooo zo handige computermacro's en je laborganisatie. Jan, al onze wederzijdse reizen tussen Nijmegen en Weesp hebben toch hun vruchten afgeworpen. Dank hiervoor.

Sylvia (mw. S. Engels), gelukkig voor mij bleken jouw DOS-systeem en mijn Macintosh computer compatibel. Ik dank je voor het met zorg verwerken van mijn ingetypte files. Bovendien wil ik je bedanken voor al je morele ondersteuning tijdens de dieptepunten die elke onderzoeker kent.

Natuurlijk wil ik ook mijn mede-ploeterende AIO's en OIO's bedanken voor de prettige sfeer die het werken veraangenaamd heeft. Leo (drs. S.L. Abrahamse), zoals een goed onderzoeker betaamd, had je mij al doorgelicht voordat ik op het Trigon verscheen. Ikzelf heb onbevooroordeeld kennis van je genomen, en ik moet zeggen dat ik onze samenwerking als plezierig ondervonden heb. Dank je voor je gezelschap tijdens het labwerk, tijdens de door ons gevolgde cursussen en tijdens onze congresbezoeken. Harold (drs. H. Raat), jij bedankt voor het optreden als "redder in nood" als een computer weer eens vast liep of gecrasht was. Henk (drs. H. Koster), mijn bord zal je missen....

Dat vele handen licht werk maken, hebben Peter Ramakers en Rianne Tijsen bewezen. Peter en Rianne, bedankt voor jullie enthousiaste inzet en jullie praktische hulp. Ik weet jullie "getel en ge-LDH" te waarderen.

Gezien ik als echte bioloog geen techneut ben, was jouw technische steun, Piet (dr. A. Vis), onontbeerlijk. Dank je voor al je hulp met de MagiCal en de

NPS, en bedankt voor het zijn van een goede collega.

Ook alle andere collega's van de afdeling Celfysiologie wil ik bedanken voor de prettige samenwerking. Ik heb jullie gezelschap altijd plezierig gevonden, en wat de taart aangaat....graag gedaan jongens!

Gedurende de afgelopen vier jaar heb ik de afdeling Fysiologie in de prekliniek, ookwel "de overkant" genoemd, niet alleen tijdens de gezamelijke maandagochtend-koffie bezocht, maar ook tijdens het opzetten van mijn zuurstofvrije model. Naast jullie gezelschap, wil ik jullie ook danken voor jullie hulp. In het bijzonder dank ik prof. dr. B. Oeseburg voor zijn inzet bij het verkrijgen van een zuurstofelectrode, Jo Michels voor zijn geduld bij het maken van onder andere een zuurstofvrij-kamertje, en Bini Ringnalda voor de vele zuurstofmetingen en haar adviezen voor zuurstofvrij werken.

Na mijn dankbetuiging in de richting van Nijmegen, wend ik me nu tot Weesp, en wel tot Solvay Duphar BV. Tijdens mijn werkbezoeken aan jullie, heb ik me altijd welkom gevoeld en ik wil me hiervoor bij jullie bedanken. Naast mijn copromotor zijn er echter nog een aantal personen tot wie ik me persoonlijk wil richten, namelijk Peter Couwenberg en Herman van Stuivenberg. Peter, jou wil ik bedanken voor de vele reizen die je 's ochtends naar Nijmegen moest maken om samen met mij de experimenten met hartspiercellen uit te voeren. Ook wil ik je bedanken voor je welbespraakte gezelschap tijdens de soms wat lang durende metingen achter de MagiCal. Herman, bedankt voor de samenwerking en de adviezen ten aanzien van de experimenten met de hartspiercellen.

Tenslotte, wil ik de voor mij belangrijkste personen bedanken, namelijk mijn ouders. Lieve vader en moeder, zonder jullie liefdevolle ondersteuning was dit proefschrift niet geworden tot wat het nu is. Altijd raapten jullie de scherven achter me op en plakten ze weer met onuitputtelijk geduld aan elkaar. Ik dank jullie hiervoor. Ook jou, Henny, wil ik bedanken. Ik kan me geen betere broer wensen. Je steunt me door dik en dun, en omdat jij me als geen ander kent, weet je me keer op keer te motiveren. Natuurlijk wil ik je ook bedanken voor jouw hulp bij het maken van de lay-out van dit proefschrift. Dat hebben we samen toch weer mooi voor elkaar gekregen.

Dank jullie wel allemaal,

Uschi

CURRICULUM VITAE

Ursula Maria Rose, roepnaam Uschi, werd op 13 januari 1966 te Deurne geboren (N-Br.). In 1984 behaalde zij het gymnasium-ß diploma aan het Mgr. Zwijsen College te Veghel. In hetzelfde jaar begon zij met de studie biologie aan de Katholieke Universiteit Nijmegen. Na het behalen van het propedeutisch examen in 1985, heeft zij het doctoraal examen afgelegd in augustus 1989, met als hoofdvak Dierfysiologie onder begeleiding van prof. dr. E.W. Roubos en dr. H.J Leenders, en als bijvak Experimentele Gynaecologie en Obstetrie onder begeleiding van prof. dr. Rolland, dr. S.L. Bastiaans en dr. H. Goverde. Tijdens haar doctoraal studie trad zij op als student-assistent bij de practica Voortplantingsfysiologie, Dierfysiologie, Ontwikkelingsbiologie en Medische Biologie. Vanaf september 1989 tot september 1993 was zij als assistent in opleiding werkzaam bij de vakgroep Celfysiologie van de Medische Faculteit aan de Katholieke Universiteit van Nijmegen. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek verricht onder begeleiding van prof. dr. C.H. van Os en dr. R.J.M. Bindels van de vakgroep Celfysiologie, en van dr. J.W.C.M. Jansen van Solvay Duphar BV te Weesp. Daarnaast leverde zij een bijdrage aan het practische onderwijs aan medische studenten. Tenslotte volgde zij de cursussen Schrijfvaardigheid Engels en Biometrie MSA, en behaalde zij in 1989 haar deskundigheid Stralingshygiëne Niveau 3 en in 1990 haar diploma Proefdierkunde.

PUBLICATIONS

U.M. Rose, R.J.M. Bindels, A. Vis, J.W C.M. Jansen and C.H Van Os (1993) The effect of L-type Ca^{2+} channel blockers on anoxia-induced increases in intracellular Ca^{2+} concentration in rabbit proximal tubule cells in primary culture. Pflugers Arch 423; 378-386

U.M. Rose, R.J.M. Bindels, J.W.C.M. Jansen and C.H. Van Os (1994) Effects of Ca^{2+} channel blockers, Ca^{2+}-free solution and glycine on cell Ca^{2+} and injury in anoxic rabbit proximal tubules. Kidney Int (in press)

U.M. Rose, J.W C.M. Jansen, R.J.M. Bindels and C.H. Van Os (1994) Effects of substrate-free anoxia and veratridine on intracellular calcium concentration in isolated rat ventricular cardiomyocytes. Pflugers Arch (in press)

U.M. Rose, S.L. Abrahamse, J.W C.M. Jansen, R.J.M. Bindels and C.H. Van Os. Cellular acidification occurs during anoxia in cultured but not in freshly-isolated rabbit proximal tubular cells. Pflugers Arch (submitted)

U.M. Rose, J.W.C.M. Jansen, R.J.M. Bindels and C.H. van Os. The role of calcium in cell injury induced by chemical anoxia in proximal tubular cells in primary culture. Cell Calcium (submitted)

U M. Rose, A. Hartog, J.W.C.M. Jansen, C.H. Van Os and R.J.M. Bindels. Anoxia-induced increases in intracellular calcium concentration in primary cultures of rabbit thick ascending limb of Henle's loop. Biochim Biophys Acta (submitted)

U.M Rose, R.J.M. Bindels, J.W.C.M. Jansen and C.H. Van Os (1991) Anoxia causes increase in intracellular calcium levels in rabbit proximal tubule cells in culture. Pharmaceutisch Weekblad Scientific edition, Supl H, Vol 13 (abstract)

U.M. Rose, C.H. Van Os, J.W.C.M. Jansen and R J.M. Bindels (1992) Anoxia-induced increase in intracellular calcium of rabbit proximal tubule cells in primary culture. Pflugers Arch Supl 1 Vol 240: R71 (abstract)

U.M. Rose, C.H. Van Os, J.W.C.M. Jansen and R.J.M. Bindels (1992) Anoxia induced increase of intracellular calcium in rabbit proximal tubule cells in primary culture Kidney Int 42: 797 (abstract)

U.M. Rose, J.W.C.M. Jansen, R.J.M. Bindels and C H. Van Os (1992) Evaluation of in vitro models of anoxia with respect to renal ischemic injury. J Am Soc Nephrol 3: 713 (abstract)

U.M. Rose, A. Vis, J.C.W.M. Jansen, C.H. Van Os and R.J.M Bindels (1992) Effects of phenylalkylamines on anoxia-induced increases in intracellular calcium in primary cultures of rabbit proximal tubular cells. J Am Soc Nephrol 3: 714 (abstract)

U.M. Rose, R.J.M. Bindels, J.W.C.M. Jansen and C.H. Van Os (1993) Protective effects of acidosis during anoxia in renal proximal tubule cells. The FASEB Journal 7: A60 (abstract)

U.M. Rose, J.W.C.M. Jansen, R.J.M. Bindels and C.H. Van Os (1993) Dissociation between elevated intracellular Ca^{2+} levels and cell death during anoxia in freshly-isolated proximal tubule cells of rabbit kidney. Pflugers Arch 424: R1-R10 (abstract)